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<p>(21) International Application Number: PCT/EP98/02070</p> <p>(22) International Filing Date: 9 April 1998 (09.04.98)</p> <p>(30) Priority Data: 97201091.2 11 April 1997 (11.04.97) EP (34) Countries for which the regional or international application was filed: NL et al.</p> <p>(71) Applicant (for all designated States except US): GIST-BROCADES B.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): SELTEN, Gerardus, Cornelis, Maria [NL/NL]; Sterrenweg 81, NL-2651 HZ Berkel En Rodenrijs (NL). SWINKELS, Bart, Willem [NL/NL]; Schutterstraat 5, NL-2611 MX Delft (NL). BOVENBERG, Roelof, Ary, Lans [NL/NL]; 's-Gravenweg 121, NL-3062 ZD Rotterdam (NL).</p> <p>(74) Agent: VOSSIUS & PARTNER; Postfach 86 07 67, D-81634 Munich (DE).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>	
<p>(54) Title: GENE CONVERSION AS A TOOL FOR THE CONSTRUCTION OF RECOMBINANT INDUSTRIAL FILAMENTOUS FUNGI</p> <p>(57) Abstract</p> <p>The present invention relates to filamentous fungi that comprise in their genomes at least two substantially homologous DNA domains which are suitable for integration of one or more copies of a recombinant DNA molecule and wherein at least two of these DNA domains comprise an integrated copy of a recombinant DNA molecule. The invention also relates to methods for preparing such filamentous fungi and for further multiplying the DNA domains with integrated recombinant DNA molecules through gene conversion or amplification.</p>			

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GENE CONVERSION AS A TOOL FOR THE CONSTRUCTION OF RECOMBINANT INDUSTRIAL FILAMENTOUS FUNGI

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Field of the invention

10 The present invention relates to genetic engineering of microorganisms used in industrial fermentation processes.

Background of the invention

15 An ever increasing number of products is produced by microbial fermentation at industrial scale. Such products range from primary and secondary metabolites, such as e.g. citric acid and antibiotics, respectively, to proteins, enzymes and even complete microorganisms, e.g. in the form
20 of baker's yeast or biomass. Traditionally, the microorganisms in question have been subjected to classical strain improvement programs which consist of successive rounds of mutagenesis and subsequent selection of mutants with improved performance. More recently, also genetic
25 engineering, i.e. recombinant DNA technology, has been applied to industrial microorganisms. This technology has not only allowed further improvement of production levels of products naturally produced by the microorganism in question, but has also allowed the development of totally
30 new products and/or production processes, such as e.g. the production of heterologous proteins or metabolic pathway engineering.

35 Genetic engineering requires the introduction and usually also the expression of a recombinant DNA molecule into the organism in question. This process is referred to as transformation. Stable transformation of a microorganism requires that the introduced recombinant DNA molecule is maintained in the cells from generation to generation, i.e.

stable inheritance. There are basically two ways of maintaining the introduced recombinant DNA molecule. First, the recombinant DNA can integrate into the host cell's genome, e.g. into one of its chromosomes. Once integrated 5 the recombinant DNA is part of this chromosome and will thus be maintained by replication together with this chromosome. Second, the recombinant DNA can be introduced into the cell as part of a DNA molecule capable of autonomous replication, i.e. replication independent of the host's genome. Such 10 autonomously replicating vectors are often derived from naturally occurring plasmids or viruses which have been adapted to accommodate the incorporation of the recombinant DNA.

15 With respect to industrial microbial production organisms the autonomous and the integrative vector systems each have their specific advantages and disadvantages. The autonomously replicating vector systems e.g. pose restrictions on the number and/or lengths of the DNA 20 sequences to be introduced, are generally considered to be less stable and usually provide lower expression levels per gene copy as compared to the integrative systems. Most importantly, however, for some very important industrial microorganisms, notably the filamentous fungi such as 25 *Aspergillus*, *Penicillium* or *Trichoderma*, stable autonomously replicating vector systems are simply not available.

With the integrative vector systems the disadvantages depend to some extent on the approach used for integration. 30 Integration into a predetermined genomic sequence through homologous recombination is difficult to combine with high copy numbers of the recombinant DNA molecule. Whereas random integration of multiple copies of the recombinant DNA molecule, as frequently applied in filamentous fungi, will 35 result in unpredictable genotypes of the transformants. Not only can this lead to a loss of advantageous properties of the transformed production strain, also the unpredictable and undefined nature of such strains is less easily accepted

by the registration authorities.

WO 91/00920 discloses yeast strains in which multiple copies of recombinant DNA molecules are integrated in the ribosomal DNA repeat cluster. The recombinant DNA molecules, in this case expression vectors for heterologous genes, additionally comprise both a deficient selectable marker gene as well as yeast ribosomal DNA sequences, the latter of which enable integration of the vectors in the ribosomal DNA repeat cluster through homologous recombination. The deficient selectable marker gene is required for selection and stable maintenance of strains containing multiple copies of the vectors integrated in the ribosomal DNA repeat cluster. In addition, WO 91/000920 suggests that multicopy integration in ribosomal DNA repeats might also be applied in fungi in general, including filamentous fungi such as *Aspergillus* species. However, the multicopy integration system disclosed in WO 91/00920 is dependent on the use of a deficient selectable marker gene. Moreover, WO 91/00920 does not provide for integration into a genomic environment which is by nature adapted to support high level RNA polymerase II transcription of protein coding genes.

Recently, ES 2 094 088 described a DNA region which is amplified in penicillin overproducing strains of *Penicillium chrysogenum* E-1 and *Penicillium chrysogenum* AS-P-78. The size of the amplified DNA region is described to be 75 and 106 kb, respectively, and contains the genes *pcbAB*, *pcbC* and *penDE* within a 16.5 kb fragment in the amplified region. It is proposed that the DNA sequences present at the left hand and right hand ends of the amplified region can be used for the construction of vectors into which marker genes have been introduced in order to promote the amplification of genetic material situated between them, in particular for obtaining strains with greater production of penicillin by random mutagenesis with nitrosoguanidine in order to increase the copy number of the vector once it has been integrated into the genome of the microorganism. However, ES

2 094 088 fails to describe whether the approach outlined above could successfully be employed. Indeed, the method described in ES 2 094 088 suffers from several drawbacks. For example, amplification of complex DNA structures occur 5 only at low frequency. Furthermore, the use of the mutagen nitrosoguanidine results in undesired spontaneous mutations in the genome of the microorganism. Moreover, mutagenic treatment can result in the deletion of the sequences of the amplified region present in the vector and, therefore, the 10 deletion of the gene of interest situated between them; see also Fierro, Proc. Natl. Acad. Sci. USA (1995), 6200-6204. Also, as described above the random integration of the vector into the genome of the microorganism will result in unpredictable genotypes of the transformant.

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Thus, the technical problem underlying the present invention is to provide a generally applicable approach for the construction of recombinant production strains of filamentous fungi that contain multiple copies of a 20 recombinant DNA molecule integrated in defined predetermined target loci in its genome and which system is not dependent on the use of a particular type of selectable marker for transformation..

25 Brief description of the drawings.**Abbreviations used in the figures:****Restriction enzymes and restriction sites:**

A = *Apa*I; Ba = *Bam*HI; B = *Bgl*III; Bs = *Bss*HII; E = *Eco*RI; H = 30 *Hind*III; K = *Kpn*I; N = *Nde*I; No = *Not*I; Ps = *Pst*I; P = *Pvu*II; Sa = *Sal*I; Sc = *Sca*I; S = *Sma*I; Sn = *Sna*BI; Spe = *Spe*I; Sp = *Sph*I; Ss = *Sst*II; Xb = *Xba*I; X = *Xho*I; Nr = *Nru*I; Hp = *Hpa*I; Sf = *Sfi*I; Ns = *Nsi*I; Bst = *Bst*XI.

35

FIGURE LEGENDS

Figure 1: Schematic view of the three *AglaA* loci in the *glaA*

DNA amplicons of an *A. niger* CBS 646.97 (deposited on April 11, 1997 at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands) transformant (ISO-505), each marked by a different restriction site (*Bam*HI, *Sal*I or *Bgl*II).
5 Truncation of each *glaA* locus differs by approximately 20 or 60 bp, to visualize each truncated *glaA* locus by the PCR-based DNA-flag test.

10 **Figure 2:** Physical maps of the *glaA* loci in parental strain *A. niger* CBS 646.97 (upper part) and the three truncated "X-marked" *glaA* loci in recombinant strain *A. niger* ISO-505 (X stands for a *Bam*HI, *Sal*I or *Bgl*II restriction site).

15 **Figure 3:** Construction pathway of the intermediate vector pGBGLA16.

20 **Figure 4:** Schematic presentation of a fusion PCR to destroy the *Kpn*I* in the 3'-*glaA* DNA sequence and to add cloning sites at the border for appropriate cloning into pGBGLA16 resulting in the intermediate vector pGBGLA18.

25 **Figure 5:** Schematic presentation of a fusion PCR to include a *Bam*HI restriction site between the 5'- and 3'-*glaA* sequences and to add cloning sites at the border for appropriate cloning into pGBGLA18 resulting in the gene-replacement vector pGBDEL5.

30 **Figure 6:** Schematic presentation of a fusion PCR to include a *Sal*I restriction site between the 5'- and 3'-*glaA* sequences and to add cloning sites at the border for appropriate cloning into pGBGLA18 resulting in the gene-replacement vector pGBDEL9.

35 **Figure 7:** Schematic presentation of a fusion PCR to include a *Bgl*II restriction site between the 5'- and 3'-*glaA* sequences and to add cloning sites at the border for appropriate cloning into pGBGLA18 resulting in the gene-replacement vector pGBDEL11.

5 **Figure 8:** Schematic presentation of hybridization patterns observed after digestion with *Kpn*I or *Bam*HI of chromosomal DNA isolated from the parent strain *A. niger* CBS 646.97 probed with the *Hind*III/*Xho*I 5'-*glaA* DNA fragment (a) or the *Sal*I/*Sal*I 3'-*glaA* DNA fragment (b).

10 **Figure 9:** Schematic presentation of hybridization patterns observed after digestion with *Kpn*I or *Bam*HI of chromosomal DNA isolated from *A. niger* CBS 646.97 transformants, wherein the linearized pGBDEL5 vector has become integrated at the *glaA* target locus via a double cross-over, probed with the *Hind*III/*Xho*I 5'-*glaA* DNA fragment (a) or the *Sal*I/*Sal*I 3'-*glaA* DNA fragment (b). In the genome of individual 15 transformants the original *Kpn*I site located within the 3'-*glaA* sequence of the host might be destroyed (K*) but not necessarily, as a consequence of the double cross-over recombination event.

20 **Figure 10:** Schematic presentation of hybridization patterns observed after digestion with *Kpn*I or *Bam*HI of chromosomal DNA isolated from the *amdS* selection marker gene free transformant *A. niger* GBA-201, comprising one "*Bam*HI-marked Δ *glaA* DNA amplicon and two remaining intact *glaA* 25 amplicons, probed with the *Hind*III/*Xho*I 5'-*glaA* DNA fragment (a) or the *Sal*I/*Sal*I 3'-*glaA* DNA fragment (b).

30 **Figure 11:** Schematic presentation of hybridization patterns observed after digestion with *Kpn*I or *Bam*HI of chromosomal DNA isolated from *A. niger* GBA-201 transformants, wherein the linearized pGBDEL9 vector has become integrated via a double cross-over, probed with the *Hind*III/*Xho*I 5'-*glaA* DNA fragment (a) or the *Sal*I/*Sal*I 3'-*glaA* DNA fragment (b). In the genome of individual 35 transformants the original *Kpn*I site located within the 3'-*glaA* sequence of the host might be destroyed (K*), but not necessarily as a consequence of the double cross-over recombination event.

5 **Figure 12:** Schematic presentation of hybridization patterns observed after digestion with *Kpn*I or *Bam*HI of chromosomal DNA isolated from the *amdS* selection marker gene free transformant *A. niger* GBA-202, comprising two "BamHI- and *Sal*I- marked" Δ *glaA* DNA amplicons and one remaining intact *glaA* amplicon, probed with the *Hind*III/*Xho*I 5'-*glaA* DNA fragment (a) or the *Sal*I/*Sal*I 3'-*glaA* DNA fragment (b).

10 **Figure 13:** Schematic presentation of hybridization patterns observed after digestion with *Kpn*I or *Bam*HI of chromosomal DNA isolated from *A. niger* GBA-202 transformants, wherein the linearized pGBDEL11 vector has become integrated via a double cross-over, probed with the 15 *Hind*III/*Xho*I 5'-*glaA* DNA fragment (a) or the *Sal*I/*Sal*I 3'-*glaA* DNA fragment (b). In the genome of individual transformants the original *Kpn*I site located within the 3'-*glaA* sequence of the host might be destroyed (K*) but not necessarily as a consequence of the double cross-over recombination event.

25 **Figure 14:** Schematic presentation of hybridization patterns observed after digestion with *Kpn*I or *Bam*HI of chromosomal DNA isolated from the *amdS* selection marker gene free transformant *A. niger* ISO-505, comprising three "BamHI-, *Sal*I- and *Bgl*II-marked" Δ *glaA* DNA amplicons, probed with the *Hind*III/*Xho*I 5'-*glaA* DNA fragment (a) or the *Sal*I/*Sal*I 3'-*glaA* DNA fragment (b).

30 **Figure 15:** Physical map of the acetamide selection marker and *glaA* targeting vector pGBAAS-1.

Figure 16: Physical map of the phytase expression and *glaA* targeting vector pGBTOPFYT-1

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Figure 17: Schematic illustration of the targeting PCR test to determine whether the *phyA* expression cassette (a) or the *amdS* cassette (b) has become targeted adjacent to one

of the 3'-3"-*glaA* target loci in *A. niger* ISO-505 transformants. Targeting of one of these cassette results in both cases in amplification of a 4.2 kb sized DNA fragment.

5 **Figure 18:** Schematic illustration of the three Δ *glaA* domains in *A. niger* ISO-505. The hybridization pattern is shown of a *Bgl*II digest probed with the 2.2 kb *Sal*II/*Xho*I 3"-*glaA* DNA fragment.

10 **Figure 19:** Schematic illustration of the Δ *glaA* domains (each marked by B^* , Sal^* and Bam^*) in pGBAAS-1/pGBTOPFYT-1 *A. niger* ISO-505 transformants, in which multiple *amdS* cassettes are targeted in one of three Δ *glaA* domains of the *A. niger* ISO-505 host. The hybridization pattern is shown of a *Bgl*II digest probed with the 2.2 kb *Sal*II/*Xho*I 3"-*glaA* DNA fragment. Note that the presence and intensity of the 7.5 kb *Bgl*II hybridizing fragment is dependent on the number (n) of integrated *amdS* cassettes.

20 **Figure 20:** Schematic illustration of the three Δ *glaA* domains (each marked by B^* , Sal^* and Bam^*) in pGBAAS-1/pGBTOPFYT-1 *A. niger* ISO-505 transformants, in which multiple *phyA* has become integrated adjacent at the 3'-3"-*glaA* target sequence of one of three Δ *glaA* domains of the *A. niger* ISO-505 host and with in addition downstream one or multiple *amdS* cassettes. The hybridization pattern is shown of a *Bgl*II digest probed with the 2.2 kb *Sal*II/*Xho*I 3"-*glaA* DNA fragment. Note that the intensity of the 7.5 kb *Bgl*II hybridizing fragment is dependent on the number (n) of integrated *phyA* and *amdS* cassettes.

35 **Figure 21:** Schematic illustration of the three Δ *glaA* domains (each marked by B^* , Sal^* and Bam^*) in pGBAAS-1/pGBTOPFYT-1 *A. niger* ISO-505 transformants, in which multiple *amdS* cassettes are integrated adjacent at the 3'-3"-*glaA* target sequence of one of three Δ *glaA* domains of the *A. niger* ISO-505 host and with in addition downstream one or

multiple *phyA* cassettes. The hybridization pattern is shown of a *Bgl*II digest probed with the 2.2 kb *Sal*I/*Xho*I 3"-*glaA* DNA fragment. Note that the intensity of the 7.5 kb *Bgl*II hybridizing fragment is dependend on the number (n) of 5 integrated *phyA* and *amdS* cassettes.

Figure 22: Schematic illustration of the three Δ *glaA* domains (each marked by B^* , SA^* and BA^*) in pGBAAS-1/pGBTOPFYT-1 *A. niger* ISO-505 transformants, in which 10 multiple *phyA* cassettes are integrated adjacent at the 3'-3"-*glaA* target sequence of one of three Δ *glaA* domains of the *A. niger* ISO-505 host and with in addition downstream one or 15 multiple *phyA* and *amdS* cassettes. The hybridization pattern is shown of a *Bgl*II digest probed with the 2.2 kb *Sal*I/*Xho*I 3"-*glaA* DNA fragment. Note that the intensity of the 7.5 kb *Bgl*II hybridizing fragment is dependend on the number (n) of integrated *phyA* and *amdS* cassettes.

Figure 23: Schematic illustration of the three Δ *glaA* domains (each marked by B^* , SA^* and BA^*) in pGBAAS-1/pGBTOPFYT-1 *A. niger* ISO-505 transformants, in which 20 multiple *amdS* cassettes are integrated adjacent at the 3'-3"-*glaA* target sequence of one of three Δ *glaA* domains of the *A. niger* ISO-505 host and in addition more downstream one or 25 multiple *amdS* and *phyA* cassettes. The hybridization pattern is shown of a *Bgl*II digest probed with the 2.2 kb *Sal*I/*Xho*I 3"-*glaA* DNA fragment.

Figure 24: Physical map of *amdS* gene free *A. niger* ISO-30 505 transformants containing one *phyA* copy (a), two *phyA* copies (b) and three *phyA* gene copies (c) targeted at the "BamHI-marked" Δ *glaA* locus. A schematic hybridization pattern is shown of *Bam*HI and *Bgl*II digests, probed with 3"-*glaA* probe.

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Figure 25: Schematic illustration of the three *glaA* domains in *A. niger* ISO-505-2 transformant, in which two

5 *phyA* cassettes are targeted at the "BamHI-marked" Δ glaA amplicon showing the "DNA-flag" genotype *BamHI*⁺/*SalI*⁺/*BglII*⁺ (A), *BamHI*²⁺/*SalI*⁻/*BglII*⁺ (B) and *BamHI*²⁺/*SalI*⁺/*BglII*⁻ (C). The hybridization patterns are shown of a *BamHI* digest probed with the 2.2 kb *SalI/XhoI* 3"-glaA DNA fragment. Note that the intensity of both *BamHI* hybridizing fragments is dependend on the presence and number of different amplicons.

10 **Figure 26:** Schematic illustration of the three glaA domains in *A. niger* ISO-505-2 transformant, in which two *phyA* cassettes are targeted at the "BamHI-marked" Δ glaA amplicon showing the "DNA-flag" genotype *BamHI*⁺/*SalI*⁺/*BglII*⁺ (A), *BamHI*²⁺/*SalI*⁻/*BglII*⁺ (B) and *BamHI*²⁺/*SalI*⁺/*BglII*⁻ (C). The hybridization patterns are shown of a *BglII* digest probed with the 1.5 kb *HindIII/XhoI* 5'-glaA DNA fragment. Note that the intensity of *BglII* hybridizing fragments is dependend on the presence and number of different amplicons.

15 **Figure 27:** Schematic illustration of the glaA domains in *A. niger* ISO-505-2 transformant, in which two *phyA* cassettes are targeted at the "BamHI-marked" Δ glaA amplicon, showing the "DNA-flag" genotype *BamHI*³⁺/*SalI*⁻/*BglII*⁻ (A), *BamHI*³⁺/*SalI*⁺/*BglII*⁻ (B) and *BamHI*⁴⁺/*SalI*⁻/*BglII*⁻ (C). The hybridization pattern is shown of a *BamHI* digest probed with the 1.5 kb *HindIII/XhoI* 5'-glaA DNA fragment. Note that the intensity of both *BamHI* hybridizing fragments is dependend on the presence and number of different amplicons.

20 **Figure 28:** Schematic illustration of the glaA domains in *A. niger* ISO-505-2 transformant, in which two *phyA* cassettes are targeted at the "BamHI-marked" Δ glaA amplicon, having the "DNA-flag" genotype *BamHI*³⁺/*SalI*⁻/*BglII*⁻ (A), *BamHI*³⁺/*SalI*⁺/*BglII*⁻ (B) and *BamHI*⁴⁺/*SalI*⁻/*BglII*⁻ (C). The hybridization pattern is shown of a *BglII* digest probed with the 2.2 kb *SalI/XhoI* 3"-glaA DNA fragment. Note that the intensity of both *BamHI* hybridizing fragments is dependend on the presence and number of different amplicons.

5 **Figure 29:** PEN amplicons in *P. chrysogenum*. Relative positions of the penicillin biosynthetic genes, HELE and HELF, within a single amplicon (between brackets). Multiple PEN amplicons are present as direct repeats (n). Note that the 3' end of HELF extends into the adjacent PEN amplicon.

10 **Figure 30:** Quantitation of PEN amplicons by Southern analyses. Relative positions of *Bst*XI sites, probes and sizes of the expected hybridization fragments from the *niaD* locus (A) and PEN amplicon (B). The amount of DNA hybridizing to the HELE probe depends on the number of PEN amplicons (n).

15 **Figure 31:** Quantitation of PEN amplicons by CHEF. Relative positions of *Not*I sites and HELE probe are indicated. The *Not*I sites are located outside the PEN amplicon. The expected sizes of hybridizing fragments depend on the number of PEN amplicons ($30 + n \times 57$) kb.

20 **Figure 32:** Targeted integration. (A) Expression vectors contain the gene of interest, regulated by a suitable promoter (P) and terminator (T). The cassette is flanked by 5" and 3" regions homologous to the target site (" and ' to distinguish vector sequence from genomic sequence, respectively). Cassette and flankings are cloned in an 25 *E. coli* vector for propagation of the expression vector. Prior to transformation, the expression vector is digested with restriction enzyme R to create a linear fragment and remove the *E. coli* sequence. (B) Integration occurs at the homologous region in the genome with the free 5' and 3' ends 30 of the transformation fragment as hot spot for recombination. (C) Resulting transformant contains 1 or multiple copies (n) of the transformation fragment (between brackets) integrated at the target site.

35 **Figure 33:** Expression vector pHELE-A1. The transformation fragment, comprising the *amdS* expression cassette flanked by 5' and 3' regions of HELE, was isolated as a *Sfi*I fragment. These sites were introduced via the oligo's used to PCR the

corresponding flankings.

Figure 34: Expression vector pHELP-A1. The transformation fragment, comprising the *amdS* expression cassette flanked by 5' and 3' regions of HELP, was isolated as a *Sfi*I fragment. These sites were introduced via the oligo's used to PCR the corresponding flankings.

Figure 35: Expression vector pHELE-E1. The transformation 10 fragment, comprising the *cefE* expression cassette flanked by 5' and 3' regions of HELE, was isolated as a *Sfi*I fragment.

Figure 36: Expression vector pHELP-F1. The transformation 15 fragment, comprising the *cefF* expression cassette flanked by 5' and 3' regions of HELE, was isolated as a *Sfi*I fragment.

Figure 37: Loss of *amdS* through recombination. (A) Direct repeats of the 5' and 3' flankings arise by tandem 20 integration of expression cassettes. (B) The 5' and 3' flankings pair and recombination occurs through a single cross-over. (C) The region between site of cross-over is lost. Note that any number of cassettes (including the genes of interest) can be lost, since all are flanked by direct repeats.

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Figure 38: Integration of *cefE* in HELE. Physical map of (A) single or (B) multiple *cefE* cassettes in HELE. Relative 30 positions of *Nru*I sites, *cefE* probe and expected sizes of hybridizing fragments are indicated. The 6.0 kb fragment occurs by multiple integrations of the cassette and its intensity depends on the number of cassettes present (n).

Figure 39: Integration of *cefE* in HELE determined by TAFE. Relative positions of *Hpa*I sites, probe and expected 35 sizes of hybridizing fragments are indicated. The size of the hybridizing fragment depends on the number (n) of cassettes integrated (5.9 + n x 6.0) kb.

Figure 40: Cassette and targeting PCR. Schematic presentation of specific template and oligo combinations. Essential for targeting PCR is that one oligo is located upstream of the 5' flanking. Hence, although several 5 cassettes can be present, only the cassette 3' of this oligo yields a PCR product. Relevant domains, oligo's and expected sizes of PCR products are indicated. (A) Cassette PCR of cefE, (B) Targeting PCR of cefE in HELE, (C) Targeting PCR of amdS in HELE, (D) cassette PCR of amdS, (E) targeting PCR 10 of cefE-amdS combination (F) targeting PCR of amdS-cefE combination (G) targeting PCR of amdS-amdS combination (H) cassette PCR of niaD locus (I) cassette PCR of cefF (J) targeting PCR of cefF integrated in HELF (K) targeting PCR of amdS integrated in HELF.

15

Figure 41: Integration of amdS in HELE. Relative positions of *Hpa*I sites, amdS probe and expected sizes of the hybridizing fragments are indicated.

20 **Figure 42:** Probes. DNA fragments isolated for preparation of probes: (A) cefE probe: *Nde*I-*Nsi*I fragment of pHEL-E1. (B) cefF probe: *Nde*I-*Nsi*I fragment of pHEL-F1. (C) amdS probe: *Not*I fragment of pHELE-A1. (D) HELE probe: *Sph*I-*Not*I fragment of pHELE-A1. (E) niaD probe: PCR product of 25 oligo's 28 and 29 with chromosomal *P. chrysogenum* DNA as template.

30 **Figure 43:** Gene conversion. Gene conversion and resulting duplication of cefE through substitution of amdS by cefE in another PEN amplicon (between brackets).

Figure 44: Integration of (cefE + cefF) in one amplicon. Relative positions of *Not*I sites, HELE robe and expected sizes of hybridizing fragments, specific for integration of 35 cefE in HELE and cefF in HELF on the same amplicon, are indicated. Note that *Not*I site are incorporated via integration of cefE and cefF cassettes.

Figure 45: Integration of *cefF* in HELF. Physical map of (A) single or (B) multiple *cefF* cassettes in HELF. Relative positions of *NruI* sites, *cefF* probe and expected sizes of hybridizing fragments are indicated. The 5.7 kb fragment 5 occurs by multiple integrations of the cassette and its intensity depends on the number of cassettes present (n).

Figure 46: Quantitation of *cefE* in (*cefE* +*cefF*) gene convertants. Relative positions of *NruI* sites, probes and 10 expected sizes of hybridizing fragments for (A) single or (B) multiple *cefE* cassettes in HELE. The 6.0 kb fragment occurs by multiple integrations of the cassette and its intensity depends on the number of cassettes present (n). (C) *niaD* locus. Gene convertants will have a hybridization 15 pattern identical to the parental strain but increased intensity relative to the *niaD* signal.

Figure 47: Quantitation of *cefF* in (*cefE* +*cefF*) gene convertants. Relative positions of *NruI* sites, probes and 20 expected sizes of hybridizing fragments for (A) single or (B) multiple *cefF* cassettes in HELF. The 5.7 kb fragment occurs by multiple integrations of the cassette and its intensity depends on the number of cassettes present (n). (C) *niaD* locus. Gene convertants will have a hybridization 25 pattern identical to the parental strain but increased intensity relative to the *niaD* signal.

Figure 48: Quantitation of *cefE* gene convertants. Relative positions of *NruI* sites, probes and expected sizes 30 of hybridizing fragments for (A) single or (B) multiple *cefE* cassettes in HELE. The 6.0 kb fragment occurs by multiple integrations of the cassette and its intensity depends on the number of cassettes present (n). (C) *niaD* locus. Gene convertants will have a hybridization pattern identical to 35 the parental strain but increased intensity relative to the *niaD* signal.

Description of the invention

In a first aspect the present invention relates to a filamentous fungus which has integrated a recombinant DNA molecule into at least two substantially homologous DNA domains of its chromosome(s), wherein the DNA domains are not the ribosomal DNA repeats. Filamentous fungi of the invention are prepared by transforming a filamentous fungus with at least two such DNA domains in its genome with a recombinant DNA molecule and identification of a transformant with at least one recombinant DNA molecule integrated in at least one of the DNA domains and by subsequent identification of strains in the progeny of the transformant, which the DNA domain comprising the recombinant DNA molecule has multiplied through gene conversion with the other versions of the DNA domain or through amplification of the DNA domain comprising the recombinant DNA molecule. In other words, the filamentous fungus of the invention has incorporated at least one recombinant DNA molecule in at least one of its endogenous DNA domains in its genome.

The filamentous fungi of the present invention are eukaryotic microorganisms and include all filamentous forms of the division Eumycota of the kingdom of fungi (see e.g. Lasure and Bennett, 1985, *Fungal Taxonomy*, In: *gene manipulations in Fungi*, pp 531-535, Academic Press, Inc.). The filamentous fungi of the present invention are morphologically, physiologically and genetically distinct from yeast: in contrast to yeasts such as *S.cerevisiae*, vegetative growth of filamentous fungi is by hyphal elongation and carbon metabolism is obligately aerobic. Moreover, yeasts like *S.cerevisiae* have a prominent stable diploid phase whereas in filamentous fungi like e.g. *Aspergillus nidulans* and *Neurospora crassa* diploids only exist briefly prior to meiosis. In addition, many industrially important filamentous fungi belong to the subdivision of Deuteromycotina, also referred to as the Fungi Imperfecti, an artificial group of fungi that are

distinguished by the absence of any known sexual form. Preferred filamentous fungi of the invention belong to the genera of *Aspergillus*, *Trichoderma*, *Penicillium*, *Cephalosporium*, *Acremonium*, *Fusarium*, *Mucor*, *Rhizophus*, 5 *Phanerochaete*, *Neurospora*, *Humicola*, *Claviceps*, *Sordaria*, *Ustilago*, *Schizophyllum*, *Blakeslea*, *Mortierella*, *Phycomyces* and *Tolypocladium*. The most preferred filamentous fungi of the invention are fungi which belong to the *Aspergillus niger* group as defined by Raper and Fennell (1965, In: The 10 genus *Aspergillus*, The Williams & Wilkins Company, Baltimore), e.g. *A.niger*, the *Aspergillus flavus* group as defined by Raper and Fennell (*supra*), e.g. *A.oryzae*, as well as the fungi *Trichoderma reesei* and *Penicillium chrysogenum*. The invention is exemplified by the filamentous fungi 15 *A.niger* and *P.chrysogenum*.

The filamentous fungi according to the invention comprise in their genome at least two substantially homologous versions of a DNA domain suitable for integration of one or more copies of a recombinant DNA molecule, wherein 20 the DNA domain is non-ribosomal DNA. In the examples herein the amplified glucoamylase (*glaA*) locus of *A.niger* and the amplified penicillin cluster of *P.chrysogenum* are used as DNA domains for integration of the recombinant DNA molecules. Both the *glaA* locus of *A.niger* and the penicillin 25 cluster of *P.chrysogenum*, comprising the penicillin biosynthetic genes *pcbAB*, *pcbC* and *penDE*, occur in a single copy in genomes of wild type *A.niger* and *P.chrysogenum* strains, respectively. Strains containing multiple copies of these DNA domains, as used in the present examples, can be 30 obtained in classical strain improvement programs by selecting for strains with improved glucoamylase or penicillin production, respectively. Frequently, such production improvements are the result of amplification of a DNA domain in the selected strains. Such amplified DNA

fact, any DNA domain of which two or more substantially homologous versions occur in the genome of a filamentous fungus can be used as long as two functional criteria are fulfilled: 1) the DNA domain should be suitable for 5 accepting the integration of a recombinant DNA molecule; 2) the DNA domain should be capable of recombination with the other substantially homologous versions of the domain in the fungal genome in order to achieve multiplication of the integrated recombinant DNA molecule through gene conversion.

10 In order to meet the first criterium, a DNA domain must be of sufficient length in order to allow targeting of the recombinant DNA molecule into the domain through homologous recombination. For this purpose a DNA domain should comprise at least 100 bp, preferably at least 1 kb and more 15 preferably at least 2 kb. The suitability of a DNA domain for integration therein of a recombinant DNA molecule is furthermore determined by the requirement that integration into the DNA domain should not disrupt functions that are essential for the viability of the filamentous fungus in 20 question.

For the second functional criterium, i.e. the capability of recombination with the other substantially homologous versions of the domain in the fungal genome, is required for 25 allowing gene conversions between the different versions of the DNA domain. The minimal requirement for this purpose is that each version of the domain is flanked on either end of the domain by DNA sequences that are sufficiently homologous to the corresponding flanking sequences of the other version of the DNA domains so as to allow homologous recombination 30 between the flanking sequences. The result of this homologous recombination being a gene conversion wherein one of the versions of the DNA domain is replaced by a duplicated version of the other DNA domain containing the integrated recombinant DNA molecule. The minimum 35 requirements regarding length and extend of homology of the flanking sequences still allowing gene conversion is not exactly known and may vary depending on the organism in question. Probably a minimum length of 100 bp with an

overall homology at least 60% will still allow gene conversion. Obviously the frequency of gene conversion will increase with increasing length and homology of the sequences flanking the DNA domain. Preferably the different 5 domains are flanked by sequences of at least 1 kb which share at least 80% homology. The fungal genome may contain one or different types of DNA domains as defined above. Examples of different types of such domains which are not perfect copies of each other are allelic variants, gene 10 families and/or genes encoding isoenzymes. Most preferred are domains which are exact copies of each other, differing at most in the presence of the integrated recombinant DNA molecule. Examples of such identical domains are amplicons. Thus, in a preferred embodiment of the invention, said DNA 15 domains suitable for integration of one or more copies of a recombinant DNA molecule are amplicons.

The overall length of the DNA domains is not important and may vary from less than 1 kb to several hundreds of kb's, e.g. in the examples herein the length of the DNA 20 domains ranges from about 57 kb per unit for the amplified penicillin cluster to more than 80 kb per unit for the amplified *glaA* locus.

The recombinant DNA molecule comprises any combination of genetic elements required to introduce a desired genetic 25 modification in the filamentous fungi of the invention. The recombinant DNA molecule comprises any genetic element, parts thereof or combinations thereof, such as a gene (coding part or complete locus), a cDNA, a promoter, a terminator, an intron, a signal sequence, any regulatory DNA 30 sequence or recognition sequence of DNA-binding proteins. The genetic elements may also include DNA sequences that have been modified i.e. containing one or more nucleotide alterations (e.g. insertions, deletions, substitutions).

The desired genetic modifications include any 35 modification, i.e. insertions, deletions, and/or substitutions of DNA sequences in a selected filamentous fungus which are the result of the introduction of one or more of the above mentioned genetic elements into the fungus

by transformation or co-transformation of the recombinant DNA molecule. It will be understood that several such genetic modifications can be introduced in independent rounds of transformation.

5 According to one embodiment of the invention, the recombinant DNA molecule integrated in the DNA domain of the filamentous fungus contains one or more expression cassettes for the expression of one or more desired genes. An expression cassette is herein understood to mean a DNA
10 fragment comprising a desired gene to be expressed and which is operably linked to the appropriate expression elements that are capable of effecting and regulating the expression of the gene. Such expression elements include promoters, signal sequences, terminators and the like.

15 If the filamentous fungus is intended for the production of proteins or enzymes either heterologous or homologous, the desired gene preferably encodes a secreted protein or enzyme and thus comprises a signal sequence effecting the secretion of the enzyme. This embodiment is e.g. exemplified
20 herein by the integration of expression cassettes comprising the *A. niger* phytase gene in the *A. niger* *glaA* amplicons. However, it will be clear to the skilled person that the invention is applicable to any protein or enzyme of interest.

25 Alternatively, if the filamentous fungus is intended for the production of primary or secondary metabolites, the desired genes will usually encode one or more intracellular enzymes that are involved in the biosynthesis of these metabolites. In a preferred embodiment according to this
30 aspect of the invention, the filamentous fungus comprises one or more recombinant DNA molecules integrated in the DNA domain, whereby the recombinant DNA molecules comprises one or more expression cassettes for intracellular enzymes that are part of a metabolic pathway which is not native to the
35 filamentous fungus. Examples of such filamentous fungi include e.g. *P. chrysogenum* strains which have integrated in their penicillin amplicons expression cassettes for a deacetoxyccephalosporin C synthetase (expandase) and a

deacetylcephalosporin C synthetase (hydroxylase), allowing these strains to synthesize adipoyl-7-aminodeacetoxycephalosporanic acid and adipoyl aminodeacetylcephalosporanic acid, respectively.

5 The mechanism by which the recombinant DNA molecule integrates in the DNA domain is not important for the invention and may depend on the application of the invention at hand. Integration of the recombinant DNA molecule in the DNA domain can occur by random integration but more 10 preferably integration occurs by homologous recombination, either by a single cross-over recombination event, i.e. resulting in an insertion of the recombinant DNA molecule, or by a double cross-over recombination event, resulting in a replacement by the recombinant DNA molecule, substituting 15 part of the original sequences of the DNA domain. In order to promote integration through homologous recombination the recombinant DNA preferably contains sequences that are homologous to the target sequences for integration in the DNA domain. Preferably such targeting sequences in the 20 recombinant DNA molecule are identical to the target sequence in the DNA domain.

In a preferred embodiment of the invention each version 25 of the substantially homologous DNA domains present in the filamentous fungus comprises an integrated copy of the recombinant DNA molecule. Full occupation with integrated recombinant DNA molecules of all the DNA domain present in the fungus produces the highest possible copy number of the recombinant DNA molecule and provides a more stable 30 situation because such a fungus contains no 'empty' DNA domains that could function as donor in gene conversions with filled DNA domains, thereby reducing the copy number of the integrated recombinant DNA molecule. Either each version of the same type of DNA domains may be occupied with 35 recombinant DNA molecules or each version of different types of said DNA domains. Preferably, each version of all types of the substantially homologous DNA domains present in the genome of the filamentous fungus comprises an integrated

copy of the recombinant DNA molecule.

In one advantageous aspect of the invention, the DNA domain used for integration of the recombinant DNA molecule is a domain which in its native state comprises an endogenous gene capable of high level expression. It is generally known that the expression level of an integrated recombinant gene can vary greatly depending on the genomic locus where that gene is integrated. The advantage of using highly expressed domains for integration of recombinant genes to be expressed is that these domains are at least capable of supporting high level expression of the endogenous gene. It is therefore likely that such domains will also support high level expression of an integrated recombinant gene. Indeed we have found that integration in the *glaA* domain of *A. niger* and integration in the penicillin cluster of *P. chrysogenum* as described in the examples herein provides higher expression levels per gene copy as compared to integration in some other genomic loci. In this context it will be understood that a gene capable of high level expression is defined as a gene which, when expressed at maximum level, produces an mRNA that constitutes at least 0.1 % of the total mRNA population, preferably at least 0.5 % of the total mRNA and most preferably at least 1 % of the total mRNA. Examples of such highly expressible endogenous genes of which the domains in which they are contained are particularly suitable for the integration of the recombinant DNA molecule of the invention are genes encoding glycolytic enzymes, amylolytic enzymes, cellulolytic enzymes and/or antibiotic biosynthetic enzymes. Even more preferred are domains comprising genes involved in industrial processes and known to be expressed at high level such as glucoamylase genes, TAKA amylase genes, cellobiohydrolase genes and penicillin biosynthetic genes.

In a further aspect of the invention, the highly expressed endogenous gene is inactivated in each copy of the DNA domain in the filamentous fungus in cases where the expression of the endogenous gene is not required. In such

cases the inactivation of the high level expression of the endogenous gene makes available energy and resources which can further the expression of the gene of interest. Moreover, in case both the desired enzyme to be produced by 5 integration of the recombinant DNA molecule and the enzyme encoded by the endogenous gene are secreted enzymes, inactivation of the endogenous enzyme will result in more pure preparations of the desired enzyme. Preferably the endogenous gene is inactivated by means of an irreversible 10 deletion of at least part of the endogenous gene in order to exclude reversion of the inactivation. More preferably the inactivation of the endogenous gene is effected by an irreversible deletion which comprises at least part of the promoter and upstream activating sequences. This is 15 particularly advantageous in cases where the expression of a desired gene encoding an enzyme to be produced by integration of the recombinant DNA molecule is driven from a promoter derived from the endogenous gene because it will eliminate competition for potentially limiting transcription 20 factors required for expression of the desired gene.

In a further embodiment of the invention, each version 25 of the DNA domains is distinguished from the other versions of the domains in the filamentous fungus by means of an unique sequence tag. Such sequence tags allow to monitor the gene conversions between the different domains which facilitates the screening and/or selection of convertants 30 with a desired genotype. Any form of sequence tags can be used as long as they allow detection of the different versions of the domain: e.g. ranging from restriction sites that are detected on Southern blots to complete selectable marker genes providing a easy assayable phenotype. A particularly useful embodiment of the sequence tag is exemplified herein and allows to detect each of the domains 35 in a single PCR using one pair of oligonucleotides to prime the PCR. The domains are modified in such a way that in the PCR each version of the domains will produce a PCR fragment with a unique length. The length and intensity of the

obtained PCR fragments are indicative for the presence and copy number of each of the domains, respectively. This form of sequence tag, referred to as "DNA flags", allows to rapidly analyze the genotype of large numbers of convertant 5 colonies, in order to obtain a convertant with the desired genotype.

The present invention further relates to methods for preparing the filamentous fungi of the invention. These 10 methods comprise the step of transforming a filamentous fungus comprising in one or more of its chromosomes at least two substantially homologous DNA domains suitable for integration of one or more copies of a recombinant DNA molecule and wherein the DNA domains are not the ribosomal 15 DNA repeats, with a recombinant DNA molecule. Transformation of filamentous fungi is nowadays routine for the skilled person and a variety of transformation protocols suitable for filamentous fungi are available.

Transformation of the filamentous fungus with the 20 recombinant DNA molecule requires the use of a selectable marker gene that allows to distinguish fungal cells that have taken up the transforming DNA form the untransformed cells. A variety of selectable marker genes are available for use in the transformation of filamentous fungi. Suitable 25 markers include auxotrophic marker genes involved in amino acid or nucleotide metabolism, such as e.g. genes encoding ornithine-transcarbamylases (*argB*), orotidine-5'-decarboxylases (*pyrG*) or glutamine-amido-transferase indoleglycerol-phosphate-synthase phosphoribosyl-30 anthranilate isomerases (*trpC*), or involved in carbon or nitrogen metabolism, such e.g. *niaD* or *facA*, and antibiotic resistance markers such as genes providing resistance against phleomycin, bleomycin or neomycin (G418). Preferably, bidirectional selection markers are used for 35 which both a positive and a negative genetic selection is possible. Examples of such bidirectional markers are the *pyrG*, *facA* and *amdS* genes. Due to their bidirectionality these markers can be deleted from transformed filamentous

fungus while leaving the introduced recombinant DNA molecule in place, in order to obtain filamentous fungi that do not contain selectable markers. This essence of this MARKER GENE FREE™ transformation technology is disclosed in EP-A-0 635 5 574, which is herein incorporated by reference. Of these selectable markers the use of dominant and bidirectional selectable markers such as acetamidase genes like the *amdS* genes of *A.nidulans*, *A.niger* and *P.chrysogenum* is most preferred. In addition to their bidirectionality these 10 markers provide the advantage that they are dominant selectable markers that, the use of which does not require mutant (auxotrophic) strains, but which can be used directly in wild type strains.

A further embodiment thus relates to filamentous fungi 15 according to the invention wherein the recombinant DNA molecule lacks a selectable marker gene, or more preferably to filamentous fungi according to the invention which altogether lack a selectable marker gene.

The selectable markers used for transforming the 20 filamentous fungi of the invention with the recombinant DNA molecule can either be physically linked to the recombinant DNA molecule to be transformed or they can be on a separate DNA molecule that is cotransformed with the desired recombinant DNA molecule. Cotransformation is routinely used 25 by those skilled in the art because it occurs at relatively high frequency in filamentous fungi.

A next step in the methods for preparing the filamentous fungi of the invention comprises the selecting of a transformant with at least one recombinant DNA molecule 30 integrated in at least one of the DNA domains of the filamentous fungus. A number of routine techniques are available to the skilled person for determining which of the obtained transformants has an integration of an recombinant DNA molecule in one of its DNA domains. In a further step 35 the selected transformant is propagated and from its progeny a strain is selected in which at least two of the DNA domains comprise the integrated recombinant DNA molecule. This means that strains are selected in which the DNA domain

comprising the integrated recombinant DNA molecule is multiplied, either through gene conversion with an "empty" DNA domain or through amplification. Such gene conversion and/or amplification events occur spontaneously at low 5 frequency. The exact frequency at which these events occur may depend on a number of variables including the fungus in question and the number, the length and the extent of homology of the DNA domains. We have found, however, that these frequencies are sufficiently high to enable one to 10 screen and select for strains in which these events have occurred using analysis techniques available today. Strains in which the DNA domain comprising the integrated recombinant DNA molecule is multiplied can e.g. be identified by simply screening for strains with higher 15 production levels of the product whose synthesis is effected by the recombinant DNA molecule, or alternatively such strains can be identified by analysing their genotype by e.g. the "DNA-flag" test as outlined above.

A method according to the invention may comprise 20 additional steps in which one of the strains in which multiplication of the DNA domain comprising the integrated recombinant DNA molecule has occurred is propagated and wherein from its progeny strains are selected in which additional copies of the DNA domains comprise the integrated 25 recombinant DNA molecule. These strains may then again be subjected to this procedure until a strain is obtained in which each of the DNA domains comprises the integrated recombinant DNA molecule. As outlined above herein, such strains provide that advantages that of high copy number of 30 the recombinant DNA molecule and improved stability.

In a further aspect of the method for preparing the filamentous fungi of the invention, the recombinant DNA molecule comprises sequences which are substantially 35 homologous to the DNA domains. The presence of such substantially homologous sequences in the recombinant DNA molecule will significantly increase the frequency at which the recombinant DNA molecule integrates into the DNA domain.

The minimum requirement for the substantially homologous sequences in the recombinant DNA molecule is not known but in practice reasonable targeting frequencies are obtained with homologous targeting sequences of at least 1 kb,
5 preferably at least 2 kb.

As mentioned above, one aspect of the invention relates to the use of bidirectional selectable markers for transforming the filamentous fungus with the recombinant DNA molecule. Once such a transformant has been obtained it is
10 advantageous to subject them to counter selection for the absence of the bidirectional marker. Such markerless transformants can then be subjected to further transformations or can be propagated to select from its progeny strains with gene conversions and/or amplifications
15 of the DNA domain comprising the recombinant DNA molecule(s).

In an alternative method for preparing a filamentous fungus according to the invention a filamentous fungus is
20 transformed with a recombinant DNA molecule and a transformant is selected with at least one recombinant DNA molecule integrated in a predetermined genomic target sequence. This transformant is subsequently propagated and from its progeny strains are selecting with at least two DNA
25 domains comprising the integrated recombinant DNA molecule. In this case the recipient filamentous fungus to be transformed with the recombinant DNA molecule does not necessarily contain multiple copies of the DNA domain. The DNA domain is rather amplified after that the recombinant
30 DNA molecule is integrated therein.

The present invention allows the preparation of recombinant filamentous fungi comprising in one or more of its chromosomes at least two substantially homologous DNA
35 domains suitable for integration of one or more copies of a recombinant DNA molecule, wherein the DNA domains are not the ribosomal DNA repeats, and wherein at least two of the DNA domains comprise an integrated copy of a recombinant DNA

molecule. These recombinant fungi can be used in processes for the production of a product of interest. Such a process will usually include the steps of culturing the recombinant cells in a medium conducive to the production of the product of interest and recovery of the product of interest from the culture medium and/or from the fungus. The products of interest can be proteins, such as an enzyme, and/or primary metabolites, such as CO_2 , alcohol or organic acids, and/or secondary metabolites, such as antibiotics or carotenoids.

10 The product of interest can also be the recombinant fungi themselves, i.e. the biomass obtained in the process. Examples of enzymes and proteins that can be produced in the filamentous fungi of the invention include lipases, phospholipases, phosphatases, phytases, proteases, 15 pullulanases, esterases, glycosidases, amylases, glucoamylases, catalases, glucose oxidases, β -glucosidases, arabinofuranosidases, rhamnosidases, apiosidases, chymosin, lactoferrin, cell wall degrading enzymes such as cellulases, hemicellulases, xylanases, mannanases, pectinases, 20 rhamnogalacturonases, and the like.

Some of the advantages of the filamentous fungi of the invention and the methods for their preparation are summarized below:

25 - The present invention provides greater versatility as compared to the available systems for non-random multicopy integration recombinant DNA molecules in filamentous fungi. Greater versatility because the present invention is not confined to the use of 30 deficient selectable marker genes for transformation, and also because the present invention is not confined to the use of only ribosomal DNA sequences as target sequence for integration.

- The filamentous fungi of the invention provide greater 35 genetic stability of the integrated multiple copies of the recombinant DNA molecule as compared to conventional recombinant filamentous fungi in which the recombinant DNA molecules are randomly integrated in tandem arrays.

In particular, filamentous fungi with a high copy number of randomly integrated recombinant DNA molecules will usually have a large numbers of tandemly repeated recombinant DNA molecules integrated in only few genomic loci. This configuration is inherently less stable than a configuration according to the invention, wherein only few tandemly repeated copies of the recombinant DNA molecule (preferably not more than five copies) are integrated in several different genomic sites such as the substantially homologous DNA domains. For example, a filamentous fungus according to the invention may have a copy number of 15, whereby the fungus may have three tandemly repeated copies of a recombinant DNA molecule integrated in each of five substantially homologous DNA domains. In contrast, a conventional recombinant filamentous fungus with a copy number of 15 may have 10 copies integrated in one undefined genomic site and 5 copies in another undefined genomic site. In accordance with the present invention it has been found the latter configuration to be less stable with respect to the loss of the recombinant DNA molecules as compared to a fungus according to the invention having the same copy number.

- The filamentous fungi of the invention are expected to provide higher expression levels per gene copy as compared filamentous fungi with integrations in ribosomal DNA because the latter has not evolved to support high level RNA-polymerase II transcription of protein encoding genes.

- The filamentous fungi of the invention are multicopy strains of which the genotype can be completely defined at least as far as the integrated recombinant DNA molecules are concerned. This will facilitate obtaining regulatory approval for processes and products in which these fungi are involved.

35 - For similar reasons the phenotype of the filamentous fungi will be much more predictable as compared to conventional recombinant filamentous fungi in which the recombinant DNA molecules are randomly integrated

because the random integration can alter the expression of unknown genes that are resident in or near the integration sites.

- The methods for preparation of the filamentous fungi of the invention provide the advantage of synergy with earlier classical strain improvement programs. As outlined above, in preferred embodiment of the invention industrial filamentous fungi are used which have been obtained in classical strain improvement programs. The use of such fungi is not only advantageous because they will often comprise amplicons that are suitable for the integration and subsequent multiplication of recombinant DNA molecules according to the invention, but also because the industrial filamentous fungi will have accumulated (a large number of) mutations that are advantageous not only for the productions of the product at which the strain improvement program was aimed (the "previous" product) but also for other new products. By modifying in these improved industrial filamentous fungi the genes involved in the production of the "previous" product so as to enable the fungus to produce a new product, the advantageous mutations will now contribute to the efficient production of the new product. In this way the present invention can save significant efforts in the development of new production strains.
- The present invention allows the "designing and building" of recombinant filamentous fungi. With the expression "designing and building" we mean that at single copy level a design is made of all the desired genetic modifications to be introduced in a DNA domain. E.g. in case of the production of a desired protein one or more expression cassettes for the protein are integrated in the DNA domain and in the same domain resident endogenous genes that might negatively influence the expression of the desired protein are inactivated. In case a mixture of proteins is to be produced the designing will comprise adjusting the expression levels of the different proteins to the

desired ratios. The latter may be particularly advantageous in the case of metabolic pathway engineering where a number of new metabolic activities, forming part of a new metabolic pathway, are to be 5 introduced in a predetermined ratio. Once these design of these desired genetic modification has been established in the DNA domain at single copy level, the building process can begin, which means that the designed single copy domain is multiplied by gene 10 conversion and/or amplification of the domain comprising the desired genetic modifications until the desired production level is reached.

EXAMPLES

5 Nomenclature

	<i>A. niger</i>	<i>Aspergillus niger</i>
	<i>P. chrysogenum</i>	<i>Penicillium chrysogenum</i>
	<i>S. clavuligerus</i>	<i>Streptomyces clavuligerus</i>
	<i>A. nidulans</i>	<i>Aspergillus nidulans</i>
10	<i>phyA</i>	<i>A. niger</i> <i>phyA</i> gene, encoding phytase.
	<i>amdS</i>	<i>A. nidulans</i> <i>amdS</i> gene, encoding acetamidase (Corrck et al., 1987 Gene 53:63-71)
	<i>cefE</i>	<i>S. clavuligerus</i> <i>cefE</i> gene, encoding deacetoxycephalosporin C synthetase. (Kovacevic et al., 1989 J. Bacteriol. 171:754-760)
15	<i>cefF</i>	<i>S. clavuligerus</i> <i>cefF</i> gene, encoding deacetylcephalosporin C synthetase. (Kovacevic, S. and Miller, J.R. 1991 J. Bacteriol. 173:398-400)
20	<i>niaD</i>	<i>P. chrysogenum</i> <i>niaD</i> gene encoding nitrate reductase (Haas et al., 1996 Biochem. Biophys. Acta 1309:81-84)
25	<i>glaA</i>	<i>A. niger</i> <i>glaA</i> gene encoding glucoamylase
	<i>gpdA</i>	<i>A. nidulans</i> <i>gpdA</i> gene, encoding glyceraldehyde 3-phosphate dehydrogenase (Punt et al., 1988 Gene 69:49-57)
30	<i>pcbC</i>	<i>P. chrysogenum</i> <i>pcbC</i> gene, encoding isopenicillin N synthase (IPNS) (Carr et al., 1986 Gene 48:257-266)
	<i>P_{gpdA}</i>	<i>gpdA</i> promoter
	<i>P_{glaA}</i>	<i>glaA</i> promoter
	<i>P_{pcbC}</i>	<i>pcbC</i> promoter
35	<i>T_{penDE}</i>	<i>penDE</i> terminator
	<i>T_{amdS}</i>	<i>amdS</i> terminator
	<i>T_{glaA}</i>	<i>glaA</i> terminator

GLA

A. niger glucoamylase protein

Abbreviations

5 CHEF Clamped Homogenous Electric Fields (electrophoresis)
TAFE Transverse Alternating Field Electrophoresis
kb kilo base
bp base pair
ADCA aminodeacetoxycephalosporanic acid
10 ADAC aminodeacetylcephalosporanic acid
oligo oligonucleotide
PCR Polymerase Chain Reaction

Oligo nucleotides:

15 1. 5'-gta gct gcg gcc gcc tcc gtc ttc act tct tcg ccc gca
 ct-3'
2. 5'-caa agg gca tgc ggc cgt atc ggc cgg tga caa aca aca tca
 ttc aac gcc-3'
3. 5'-atg ttt aag ctt ggc cga tac ggc caa aac acc ttt gat
20 ttc-3'
4. 5'-caa gtt gcg gcc gct cct cac taa cga gcc agc aga tat
 cga tgg-3'
5. 5'-aag ctt atg cgg ccg cga att cga gct ctg tac agt gac-
 3'
25 6. 5'-cg^g tac gtg cgg ccg ctc gta cca tgg gtt gag tgg tat
 g-3'
7. 5'-ata tgt gcg gcc gct tta cat ggt caa tgc aat tag atg
 gtg g-3'
8. 5'- ata act cta gag gcc cta ccg gcc ttt gca aat ata ctg
30 taa gaa cc-3'
9. 5'-gta tat tct gca ggg ccg gta ggg cca aca gtt tcc gca
 ggt g-3'
10. 5'-gta tgg gcg gcc gct tta caa cta gaa tat ggg aac ctg
 tgg g-3'
35 11. 5'-ctc gag tgc ggc cgc aaa gct agc ttg ata tcg aat tcc
 tta tac tgg gcc tgc tgc att g-3'
12. 5'-gtc cat atg ggt gtc tag aaa aat aat ggt gaa aac ttg
 aag gcg-3'

13. 5'-cat atg gcg gac acg ccc gta ccg atc ttc-3'
14. 5'-atg cat tgg ctc gtc atg aag agc cta tca tcc ggc ctg
cgg ctc gtt ctt cgc-3'
15. 5'-cag cta ccc cgc ttg agc aga cat c-3'
- 5 16. 5'-gtc agg gaa gaa cac gag ggc gca g-3'
17. 5'-ccc tct ctt cgt cgt tgt cca cgc c-3'
18. 5'-atg tcc ttg gcc gac ttc agc tcg g-3'
19. 5'-gac gag cca atg cat ctt ttg tat g-3'
20. 5'-cg^g gta ctc gct cta cct act tcg g-3'
- 10 21. 5'-gcc cag tat aag gaa ttc gat atc aag-3'
22. 5'-agg gtc gac act agt tct aga gcg g-3'
23. 5'-gac gtt atc gga cgg aga ctc agt g-3'
24. 5'-gcc tac tct gtt ctg gag agc tgc-3'
25. 5'-ccc cca tcc cgg tca cgc act cgc g-3'
- 15 26. 5'-cac aga gaa tgt gcc gtt tct ttg g-3'
27. 5'-tca cat atc ccc tac tcc cga gcc g-3'
28. 5'-gtc gcg tat ccc agg-3'
29. 5'-gtc aaa gga tat gca tac-3'
30. 5'-agc tta tgc ggc cgc gaa ttc agg tac cgt atc tcg aga-
3'
- 20 31. 5'-aat ttc tcg aga tac ggt acc tga att cgc ggc cgc ata-
3'
32. 5'-gtg cga ggt acc acc aca atc aat cca ttt cgc-3'
33. 5'-atg gtt caa gaa ctc ggt agc ctt ttc ctt gat tct-3'
- 25 34. 5'-aga atc aag gaa aag gct acc gag ttc ttg aac cat-3'
35. 5'-atc aat cag aag ctt tct ctc gag acg ggc atc gga gtc
ccg-3'
36. 5'-gac cat gat tac gcc aag ctt-3'
37. 5'-gga tcc tta act agt taa gtg ggg gcc tgc gca aag-3'
- 30 38. 5'-tta act agt taa gga tcc aca atc aat cca ttt cgc-3'
39. 5'-gct cta gag cgg ccg cga att cat ccg gag atc c-3'
40. 5'-ctt tgc gca ggc ccc cac-3'
41. 5'-tgc agg gta aat cag gga-3'
42. 5'-tcc gct aaa ggt ggt cgc g-3'
- 35 43. 5'-ccc cag cat cat tac acc tc-3'
44. 5'-aaa gga ccc gag atc cgt ac-3'
45. 5'-tct cga tac caa ggt cac cac ggg c-3'
46. 5'-gca tcc atc ggc cac cgt cat tgg a-3'

47. 5'-atc cag acc agc aca ggc agc ttc g-3'
48. 5'-tcc gca tgc cag aaa gag tca ccg g-3'
49. 5'-gtc gac tta act agt taa ggc ttc aga cgc agc gag-3'
50. 5'-tta act agt taa gtc gac aca atc aat cca ttt cgc-3'
51. 5'-aga tct tta act agt taa gtg gcc tga aca gtg ccg-3'
52. 5'-tta act agt taa aga tct aca atc aat cca ttt cgc-3'

Materials & Methods**General procedures**

Standard molecular cloning techniques such as DNA isolation, gel electrophoresis, enzymatic restriction modifications of nucleic acids, Southern analyses, *E.coli* transformation e.a., were performed as described by Sambrook *et al.* (1989) "Molecular Cloning: a laboratory manual", Cold Spring Harbor Laboratories, Cold Spring Harbor, New York and Innis *et al.* (1990) "PCR protocols, a guide to methods and applications" Academic Press, San Diego. Synthetic oligo deoxynucleotides were obtained from ISOGEN Bioscience (Maarssen, The Netherlands). DNA sequence analyses were performed on an Applied Biosystems 373A DNA sequencer, according to supplier's instructions.

Transformation of *Aspergillus niger*.

Transformation of *A. niger* was performed according to the method described by Tilburn, J. *et.al.* (1983) *Gene* 26, 205-221 and Kelly, J. & Hynes, M. (1985) *EMBO J.*, 4, 475-479 with the following modifications:

Spores were grown for 16 hours at 30°C in a rotary shaker at 300 rpm in *Aspergillus* minimal medium. *Aspergillus* minimal medium contains per liter: 6 g NaNO₃; 0.52 g KCl; 1.52 g KH₂PO₄; 1.12 ml 4 M KOH; 0.52 g MgSO₄.7H₂O; 10 g glucose; 1 g casaminoacids; 22 mg ZnSO₄.7H₂O; 11 mg H₃BO₃; 5 mg FeSO₄.7H₂O; 1.7 mg CoCl₂.6H₂O; 1.6 mg CuSO₄.5H₂O; 5 mg MnCl₂.2H₂O; 1.5 mg Na₂MoO₄.2H₂O; 50 mg EDTA; 2 mg riboflavin; 2 mg thiamine-HCl; 2 mg nicotinamide; 1 mg pyridoxine-HCL; 0.2 mg pantothenic acid; 4 µg biotin; 10 ml Penicillin (5000 IU/ml) Streptomycin (5000 UG/ml) solution (Gibco).
- Novozym 234 (Novo Industries) instead of helicase was used for preparations of protoplasts;
- after protoplast formation (60-90 minutes), KC buffer (0.8 M KCl, 9.5 mM citric acid, pH6.2) was added to a final volume of 45 ml, the protoplast suspension was centrifuged for 10 minutes at 3000 rpm at 4°C in a swinging-bucket rotor. The protoplasts were resuspended in 20 ml KC buffer

and subsequently 25 ml of STC buffer (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 50 mM CaCl₂) was added. The protoplast suspension was centrifuged for 10 minutes at 3000 rpm at 4°C in a swinging-bucket rotor, washed in STC-buffer and 5 resuspended in STC-buffer at a concentration of 10⁸ protoplasts/ml;

- to 200 µl of the protoplast suspension the DNA fragment, dissolved in 10 µl in TE buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA), 100 µl of a PEG solution (20% PEG 4000 (Merck), 10 0.8 M sorbitol, 10 mM Tris-HCl pH 7.5, 50 mM CaCl₂) was added;

- after incubation of the DNA-protoplast suspension for 10 minutes at room temperature, 1.5 ml PEG solution (60% PEG 4000 (Merck), 10 mM Tris-HCl pH 7.5, 50 mM CaCl₂) was added 15 slowly, with repeated mixing of the tubes. After incubation for 20 minutes at room temperature, suspensions were diluted with 5 ml 1.2 M sorbitol, mixed by inversion and centrifuged for 10 minutes at 4000 rpm at room temperature. The protoplasts were resuspended gently in 1 ml 1.2 M 20 sorbitol and plated onto selective regeneration medium consisting of *Aspergillus* minimal medium without riboflavin, thiamine.HCL, nicotinamide, pyridoxine, pantothenic acid, biotin, casaminoacids and glucose, supplemented with 10 mM acetamide as the sole nitrogen 25 source, 1 M sucrose, solidified with 2% bacteriological agar #1 (Oxoid, England). After incubation for 6-10 days at 30°C, the plates were replica plated onto selective acetamide plates consisting of *Aspergillus* minimal medium with 2% glucose instead of sucrose and 1.5% agarose instead 30 of agar. Single transformants were isolated after 5-10 days of growth at 30°C.

Transformation of *Penicillium chrysogenum*.

35 The Ca-PEG mediated protoplast transformation procedure is used. Preparation of protoplasts and transformation of *P. chrysogenum* was performed according to the method described

by Gouka et al., Journal of Biotechnology 20 (1991), 189-200 with the following modifications:

- After transformation, the protoplasts were plated onto selective regeneration medium plates consisting of
- 5 *Aspergillus* minimal medium, osmotically stabilized with 1.2 M sucrose, containing 0.1% acetamide as sole nitrogen source and solidified with 1.5% bacteriological agar #1 (Oxoid, England).
- After 5-8 days of incubation at 25°C transformants
- 10 appeared.

TAFE

DNA fragments ranging from 20 to 50 kb were separated on 1% agarose gels by TAFE, using a Beckman Geneline™ II apparatus (Beckman) according to instructions of the suppliers. Electrophoresis parameters: 4 sec A, 4 sec B pulsetimes at 300 mA for approximately 18 h at 14°C.

CHEF

20 DNA fragments larger than approximately 50 kb were separated on 1% agarose gels by CHEF electrophoresis, using a CHEF-DRII module (BioRad), equipped with a Pulsewave 760 Switcher, Model 200/2.0 power supply, and CHEF electrophoresis cell. Electrophoresis parameters: initial 25 time 50 sec, final time 90 sec, start ratio 1.0, 200 V for 20 to 24 h at 14°C.

Quantitation of PCR products and hybridization signals

PCR products and hybridization signals were quantitated 30 from photographs of ethidium bromide stained agarose gels or autoradiograms, respectively, according to the ImageQuaNT™ software (Molecular Dynamics).

DNA labeling and hybridizations

35 DNA labeling and hybridizations were according to the ECL™ direct nucleic acid labeling and detection systems. (Amersham LIFE SCIENCE, Little Chalfont, England).

Cassette- and DNA-flag specific PCR procedure on *A. niger* colonies.

5 *A. niger* spores were plated onto PDA-plates (Potato Dextrose Agar, Oxoid; prepared according to the supplier's instructions). After growth for 48 hours at 30°C, the $\frac{1}{2}$ - $\frac{1}{4}$ part mycelium of a single colony was transferred to 50 μ l novozym (5 mg Novozym 234 (Novo Industries) per ml KC (0.8 M KCl, 9.5 mM citric acid, pH 6.2)) and incubated for 10 10 hours at 37°C. Subsequently, 300 μ l DNA dilution buffer (10 mM Tris-HCl pH 7.5; 10 mM NaCl; 1 mM EDTA) was added and the suspension was boiled for 5 minutes at 100°C, followed by vigorously fortexing to rupture intact mycelium. 5 μ l of these mixtures was used as template in 50 μ l PCR reactions 15 containing 5 μ l 10 x Super Taq PCR buffer 1 (HT Biotechnology Ltd.), 8 μ l dNTP's (1.25 mM each), 20 - 80 ng of each oligo nucleotides and 1U Super Taq (HT Biotechnology Ltd, Cambridge, UK). The optimal amount of oligo's was determined experimentally for each purchased 20 batch.

Twenty-five amplification cycles, (each: 1 min 94°C, 1 min 55°C and 1.5 min 72°C) and a final extension step of 7 min 72°C, were performed in a DNA-amplifier (e.g. Perkin-Elmer, Hybaid).

25 For the DNA flag-test, as primer set oligo's 40/41 was used, for the *phyA* cassette PCR test oligo set 42/43 and for the *amdS* cassette test the oligo set 15/16. Subsequently, to analyse PCR products, 20 μ l of the PCR mixture was analyzed by agarose gel electrophoresis on a 2% 30 agarose gel in TBE buffer (0.09M Tris, 0.09 M H₃BO₃, 2 mM EDTA; pH 8.3).

Targeting specific PCR procedure for *Aspergillus niger*.

Preparation of DNA-templates and PCR reaction conditions 35 were used as described for the targeting PCR test for *Penicillium*. As primer sets, oligo's 46/48 were used to determine whether the *amdS* cassette is targeted adjacent to

the *glaA* target locus, and oligo set 46/47 to determine whether the *phyA* cassette is targeted adjacent to the *glaA* target locus. Used PCR conditions were as described for the targeting PCR test for *Penicillium*.

5

Targeting PCR of *P. chrysogenum* transformants

Approximately one third of 4-days old colonies was incubated for 2 h at 37°C in 50µl KC buffer (60 g/l KCl, 2 g/l citric acid, pH 6.2), supplemented with 5 mg/ml Novozym™234. Subsequently 100 µl 10mM Tris, 50mM EDTA, 150mM NaCl, 1% SDS, pH8 and 400 µl QIAquick™ PB buffer (Qiagen Inc., Chatsworth, USA.) was added. Extracts were gently resuspended and loaded onto a QIAquick™ spin column. Columns were centrifugated for 1 min at 13000 rpm in a microfuge and washed once with 500 µl QIAquick™ PE buffer. Traces of ethanol were removed by a final quick spin. Chromosomal DNA (PCR template) was eluted from the column by addition of 50µl H₂O and subsequent centrifugation for 1 min at 13000 rpm. PCR reactions contained 10µl eLONGase™ B buffer (Life Technologies, Breda, The Netherlands), 14µl dNTP's (1.25 mM each), 1µl eLONGase™ Enzyme Mix, 1µl template, and 30-50 ng of each oligo, in a final volume of 50µl. The optimal amount of oligo's was determined experimentally for each purchased batch. On average, 30 to 50 ng was used. Reactions were performed with the following cycle conditions: 1x (2 min 94°C), 10x (15 sec 94°C, 30 sec 55°C, 4 min 68°C), 20x (15 sec 94°C, 30 sec 55°C, 4 min start with incline of 20 sec per cycle, 68°C), 1x (10 min 68°C). Samples of 8µl were loaded on agarose gels for analyses of PCR products.

Cassette PCR of *P. chrysogenum* transformants

Approximately one third of 4-days old colonies was incubated for 2 h at 37°C in 200µl DVB buffer (10 mM Tris, 10 mM NaCl, 1 mM EDTA, pH7.5), supplemented with 5mg/ml Novozym™234. The mixture was boiled for 8 min and

subsequently vortexed vigorously to disrupt intact mycelia. Five μ l of these mixtures was used as template in 50 μ l PCR reactions containing 5 μ l 10 x Super Taq PCR buffer (HT Biotechnology Ltd., Cambridge, UK), 8 μ l dNTP's (1.25 mM each), 1 U Super Taq and 20-80 ng of each oligo. The optimal amount of oligo's was determined experimentally for each purchased batch. On average, 20 to 80 ng was used. Reactions were performed with the following cycle conditions: 25 x (1 min 94°C, 1 min 55°C, 1,5 min 72°C), 1 x (8 min 72°C). Samples of 15 μ l were loaded on agarose gels for analyses of PCR products.

Chromosomal DNA isolation.

A. niger and *P. chrysogenum* spores were inoculated in 20 ml 15 *Aspergillus* minimal medium (see transformation of *A. niger*) in a 100 ml conical flask and grown for 20-24 hours at 30°C in a rotary shaker at 300 rpm. 5-10 ml of a well grown culture were inoculated in 100 ml fresh *Aspergillus* minimal medium and grown for another 20-24 hours at 30°C in a rotary 20 shaker at 300 rpm. After growth, mycelium was harvested by filtration over a Miracloth (Cal Biochem) filter and washed with 15 ml KC buffer (0.8 M KCl, 9.5 mM citric acid, pH 6.2). Per gram of the washed mycelium (3-5 g. of a 100 ml culture) 4 ml KC buffer and 0.25 ml novozym (50 mg Novozym 25 234 (Novo Industries) /ml KC buffer) was added and protoplast formation was allowed to take place at 30°C under gently shaking. After protoplast formation, KC buffer was added to a volume of 45 ml and the protoplast suspension was centrifuged at 3000 rpm at 4°C for 5 minutes in a 30 swinging bucket rotor. Protoplasts were resuspended in 20 ml KC buffer. Then 25 ml of STC buffer (1.2 M sorbitol, 10 mM Tris-HCl pH 7.5, 50 mM CaCl₂) was added and after mixing the suspension was subsequently centrifuged at 3000 rpm at 4°C for 5 minutes in a swinging bucket rotor, resuspended in 35 50 ml STC buffer and again centrifuged for 5 minutes at 2500 x g at 4°C in a swinging bucket rotor. To 0.3-0.6 ml of the protoplast pellet 60 μ l proteinase-K (20 mg/ml) and 1 ml

low melting point agarose (0.8% in 1 M Sorbitol/0.45 M EDTA) was added. After mixing by vortexing, the suspension was transferred to wells of a preformed plexiglass mold and set on ice for approx. 15 minutes. Next, the agarose plugs 5 were transferred to 2.5 ml sarcosyl solution (1% Na-lauroyl sarcosine in 0.5 M EDTA). 60 μ l Proteinase-K (20 mg/ml) was added and after incubation for 16-20 hours at 50°C the sarcosyl solution was replaced by 10 ml 50 mM EDTA solution. After 2 hours incubation at 50°C, the EDTA 10 solution was replaced by 10 ml fresh 50 mM EDTA and incubated for another 2 hours at 50°C. Finally, the EDTA solution was replaced by 10 ml fresh 50 mM EDTA solution and the agarose plugs were stored at 4°C.

15 **Restriction enzyme digestions of chromosomal DNA agarose plugs.**

Part of a DNA agarose plug (prepared as described above), containing 1-3 μ g chromosomal DNA, was incubated in 1 ml TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) for 1 hour at 37°C. The 1 20 ml TE-buffer was refreshed and the incubation continued for another hour at 37°C. Then the TE-buffer was replaced by 200 μ l of a buffer appropriate for each restriction enzyme as recommended by the supplier. After an incubation at 37°C for one hour, the restriction enzyme buffer was 25 refreshed (50 μ l) and incubated for 10 minutes at 65°C to dissolve the agarose. Finally, 20-40 Units of the appropriate restriction enzyme was added and the digestion mixture was incubated for 16 hours at a temperature as recommended by the supplier for each restriction enzyme.

AmdS counter selection procedure.

In most derived vectors the *amdS* selection marker gene is positioned between DNA repeats. Therefor, in realized transformants with these vectors, removal of the *amdS* marker gene can be achieved either by internal recombination over the flanking DNA repeats within the cassette or by homologous recombination over repeats that are created by integration via a single cross-over event. Selection of cells that have lost the *amdS* selection marker is achieved by growth on plates containing fluoroacetamide. Cells harboring the *amdS* gene metabolize fluoroacetamide to ammonium and fluoroacetate which is toxic to the cell. Consequently, only cells that have lost the *amdS* gene are able to grow on plates containing fluoroacetamide.

In case of removal of the *amdS* marker from *Aspergillus* transformants, spores from these transformants were plated onto selective regeneration medium (described above) containing 5 mM fluoroacetamide and 5 mM ureum instead of 10 mM acetamide, 1.1% glucose instead of 1M sucrose and 1.1% instead of 2% bacteriological agar #1 (Oxoid, England). After 7-10 days of growth at 30°C single colonies were harvested and plated onto 0.4% potato dextrose agar (Oxoid, England).

In case of removal of the *amdS* marker from *Penicillium* transformants, spores from these transformants were plated onto *Aspergillus* minimal medium containing 10 mM fluoroacetamide and 5% glucose, solidified with 1.5% bacteriological agar #1 (Oxoid, England). After 5-10 days of growth at 25°C, resistant colonies appeared.

***E. coli* bioassay**

Transformants were grown on YPD agar medium for 5 days. *E.coli* ESS2231 was used as indicator bacterium in an agar overlay, that also contained Bacto penase to be able to 5 discriminate between penicillin and cephalosporin production, according to methods well known in the art (Guttiérez et al., Mol. Gen. Genet. 1991 225:56-64). Transformants expressing *cefE* were identified by a clarification of the agar overlay around the colony.

10

Industrial strain improvement of P. chrysogenum

Standard mutagenic techniques and screening procedures well known to persons skilled in the art (Rowlands, 1984 Enzyme Microb. Technol. 6:3-10) were used to isolate 15 *P. chrysogenum* strain CBS 649.97 (deposited on April 11, 1997 at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands) from Wis54-1255 (ATCC 28089)

Adipoyl-7-ADCA and adipoyl-7-ADAC fermentations

20 Fermentative productions and quantitation of adipoyl-7-ADCA and adipoyl-7-ADAC were essentially as described for production of 2-(carboxyethylthio) acetyl- and 3-(carboxymethylthio) propionyl-7-ADCA (WO 95/04148) with the exception that 3 g/l adipic acid was added to the culture 25 medium instead of 3'- carboxymethylthiopropionic acid. Synthetic adipoyl-7-ADCA and adipoyl-7-ADAC were used as reference substrates.

***Aspergillus niger* shake flask fermentations.**

30 Of recombinant and control *A. niger* strains a large batch of spores were generated by plating spores or mycelia onto PDA-plates (Potato Dextrose Agar, Oxoid), prepared according to the supplier's instructions. After growth for 3-7 days at 30 °C spores were collected after adding 0,01% 35 Triton X-100 to the plates. After washing with sterile water about 10⁷ spores of selected transformants and control strains were inoculated into shake flasks, containing 20 ml of liquid preculture medium containing per liter: 30 g

maltose.H₂O; 5 g yeast extract; 10 g hydrolyzed casein; 1 g KH₂PO₄; 0.5 g MgSO₄.7H₂O; 0.03 g ZnCl₂; 0.02 g CaCl₂; 0.01 g MnSO₄.4H₂O; 0.3 g FeSO₄.7H₂O; 3 g Tween 80; 10 ml penicillin (5000 IU/ml)/Streptomycin (5000 UG/ml); pH 5.5.

5 These cultures were grown at 34°C for 20-24 hours. 10 ml of this culture was inoculated into 100 ml of *A. niger* fermentation medium containing per liter: 70 g maltodextrines; 25 g hydrolyzed casein; 12.5 g yeast extract; 1 g KH₂PO₄; 2 g K₂SO₄; 0.5 g MgSO₄.7H₂O; 0.03 g ZnCl₂; 0.02 g

10 CaCl₂; 0.01 g MnSO₄.4H₂O; 0.3 g FeSO₄.7H₂O; 10 ml penicillin (5000 IU/ml)/Streptomycin (5000 UG/ml); adjusted to pH 5.6 with 4 N H₂SO₄. These cultures were grown at 34°C for 6 days. Samples taken from the fermentation broth were

15 centrifuged (10', 5.000 rpm in a swinging bucket centrifuge) and supernatants collected. Glucoamylase or phytase activity assays (see below) were performed on these supernatants..

Glucoamylase activity assay.

20 The glucoamylase activity was determined by incubating 10 µl of a six times diluted sample of the culture supernatant in 0.032 M NaAC/HAC pH 4.05 with 115 µl of 0.2% (w/v) p-Nitrophenyl α-D-glucopyranoside (Sigma) in 0.032 M NaAc/HAc pH 4.05. After a 30 min incubation at room temperature, 50

25 µl of 0.3 M Na₂CO₃ was added and the absorbance at a wavelength of 405 nm was measured. The A_{405nm} is a measure for the GLA production.

Phytase activity assay.

30 100 µl supernatant (diluted when necessary) of shake flask- or micro-titer *Aspergillus niger* fermentations (as reference 100 µl demiwatet) is added to 900 µl mixture, containing 0.25 M sodium acetate buffer pH 5.5, 1 mM phytic acid (sodium salt, Sigma P-3168) and incubated for 30

35 minutes at 37°C. The reaction is stopped by the addition of 1 ml 10% TCA (trichloroacetic acid). After the reaction has been terminated, 2 ml of reagent (3.66 g FeSO₄.7H₂O in 50 ml

of ammonium molybdate solution (2.5 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ and 8 ml H_2SO_4 diluted up to 250 ml with demiwater) is added. The absorbance of the blue color is measured spectrophotometrically at 690 nm. The measurements are 5 indicative of the quantity of phosphate released in relation to a calibration curve of phosphate in the range of 0-1 mMol/l.

Culturing of microorganisms

10 *S. clavuligerus* strain ATCC 27064 was cultured at 27°C in tryptic soy broth (Difco).

P. chrysogenum strains were cultured at 25°C in complete YPD medium (1% yeast extract, 2% peptone, 2% glucose) For solid media, 2% Bacto-agar was added. Transformants were purified 15 by repeated cultivation on YPD agar. Single stable colonies were used to prepare agar slants for production of spores.

20 *A. niger* strains were cultured at 30°C on PDA containing plates. Transformants were purified by repeated cultivation on PDA plates. Single stable colonies were used to prepare again slants for production of spores.

E. coli strains were cultured according to standard procedures (Sambrook)

25

EXAMPLES A. NIGER

1.1 Selection and characterization of *Aspergillus niger* hosts.

30 **1.1.a Rationale.** The presence of repeated DNA domains in the genome is essential to use gene conversion as tool to amplify inserted recombinant expression cassettes. For example, the *glaA* gene of high glucoamylase producing *A. niger* strains could be present on such amplified DNA 35 domains. In order to identify suitable hosts for the production of industrial enzymes we screened for *A. niger* strains with improved glucoamylase production. Here we describe the procedure how to select and to characterize

such *A. niger* strains containing the required DNA amplicons.

1.1.b **Selection of *A. niger* mutants.** From the *A. niger* CBS 513.88 (deposited October 10, 1988) several mutant strains were selected showing an improved production of glucoamylase on shake flask level. Mutagenesis was performed on spores of *A. niger* CBS 513.88 by UV-treatment. Surviving spores (approximately 1%) were tested for their ability to produce glucoamylase in shake flask fermentations as described in Material & Methods. After 6 days of growth glucoamylase enzyme activities were determined as described in Material & Methods.

Several *A. niger* mutant strains could be selected showing enhanced glucoamylase production levels even up to 600 U glucoamylase/ml. The parent strain *A. niger* CBS 513.88 reaches a level of approximately 200 glucoamylase U/ml. The best producing *A. niger* mutant strain was deposited as *A. niger* CBS 646.97 on April 11, 1997 at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands.

1.1.c **Genetic characterization of *A. niger* CBS 646.97.** To determine whether the above described increased in glucoamylase production is the consequence of an amplification of the *glaA* locus, chromosomal DNA was isolated from the mutant strain *A. niger* CBS 646.97 and its parent *A. niger* CBS 513.88. Southern analyses were carried out on isolated DNA, digested with *Eco*RI and *Sal*I and probed with a glucoamylase/phytase DNA fragment (*glaA/phyA* fusion: an *Eco*RI/ *Bam*HI fragment from pFYT3, which is described in Patent Application EP 0420358A1)). Autoradiographs clearly show that improved glucoamylase mutant strain contains multiple (3-4) *glaA* gene copies.

35 1.1.d. **Size of *glaA* amplicons in the mutant *A. niger* strain.** TAFE analyses revealed that large DNA fragments are amplified in the selected mutant *A. niger* strain. Chromosomal DNA was derived from the original *A. niger* CBS

513.88 strain as well as from the *A. niger* CBS 646.97 strain as described in Materials & Methods. Digestions with *Hind*III and the eight-cutter restriction enzymes *Not*I, *Swa*I, *Asc*I and *Pac*I were done and subsequently supplied to 5 TAFE-analyses using several *glaA* locus specific probes derived from pAB6-1 (see Patent Application EP 0357127A1). No differences could be observed with respect to the size of the *glaA* hybridizing DNA fragments with the above mentioned restriction enzymes between the parent and the 10 mutant strain. The largest hybridizing DNA fragment could be detected in all cases with the restriction enzyme *Swa*I: approximately 80 kb. The only observed difference is the intensity of the hybridizing *glaA* band. Although the intensity of the hybridizing *glaA* bands is difficult to 15 quantify accurately, the TAFE-autoradiographs clearly shows that the mutant strain *A. niger* CBS 646.97 contains additional copies of the observed *glaA* amplicon. In addition, we couldn't find another restriction enzyme which cuts outside the detected *glaA* amplicon. This means 20 that the *glaA* amplicon encompasses at least 80 kb in size or larger.

1.2 Modifications of *glaA* loci on the various amplicons of 25 *A. niger* CBS 646.97 strain.

1.2.a. **Rationale.** As described previously (Patent Application EP 0635574A1) a genomic target gene can be deleted appropriately in *A. niger* by using the "MARKER-GENE FREE" approach. In that patent application an example is 30 extensively described how to delete *glaA* specific DNA sequences in the genome of *A. niger* CBS 513.88. Described replacement vector integrates into the *A. niger* *glaA* genomic sequence via a double cross-over homologous recombination. The deletion vector comprises DNA regions homologous to the 35 *glaA* target locus and the *amdS* selectable marker gene driven by the *gpdA* promoter sequence. In addition, in this vector the *amdS* selectable marker gene is flanked at both sites by *glaA* sequences as direct DNA repeats to facilitate

subsequently the appropriate elimination of the *amdS* marker gene.

Such vectors direct replacement of the *glaA* gene by the *amdS* marker gene. Subsequently, by performing the 5 fluoroacetamide counter selection on these transformants, the *amdS* marker gene can be deleted properly by an internal recombination event between the 3'-*glaA* DNA direct repeats. This resulted in a MARKER-GENE FREE Δ *glaA* recombinant *A. niger* CBS 513.88 strain, possessing finally no foreign DNA 10 sequences at all.

It's obvious that this "MARKER-GENE FREE" approach is especially useful for modifying and deleting host genomic sequences repeatedly.

This example describes the appropriate deletion of all *glaA* 15 promoter and encoding sequences in *A. niger* CBS 646.97, in three successive transformation rounds by using a set of three "special designed" pGBDEL replacement vectors. For these three deletion rounds a set of three more or less the same *glaA* gene-replacement pGBDEL vectors were constructed 20 and used. The reason to include in each pGBDEL vector an unique restriction enzyme recognition DNA sequence, is to recognize and visualize in derived Δ *glaA* recombinant *A. niger* strain each truncated *glaA* locus separately by Southern analyses (described in example 1.2.e). The unique 25 restriction site in each pGBDEL vector is positioned at the 5'-border of the truncated *glaA* locus. However, in each pGBDEL vector, the included *glaA* sequences for directing targeting of the gene-replacement vector at one of the *glaA* loci, is slightly different. The consequence is that each 30 truncated *glaA* locus in the *A. niger* host, is slightly different as well. The reason to include this slight difference is to visualize each truncated *glaA* locus by a rapid PCR test on colonies: the so-called "DNA-flag" test (figure 1). Although not essential, this incorporated DNA 35 feature is especially helpful to monitor, with aid of the in this application described phenomenon of gene conversion events between the *glaA* amplicons in *A. niger*, the "building" process to amplify the (phytase) expression

5 cassettes, which are targeted at one of these truncated *glaA* loci of the *A. niger* host. Details of this "building" process to derive rDNA strains for the production of enzymes, making use of the conversion events between *glaA* amplicons in *A. niger* is described below (examples 1.5 and 1.6).

10 **1.2.b Description of *glaA* replacement pGBDEL vectors.** All constructed pGBDEL replacement vectors comprises:

- a 2 kb sized 5'-*glaA* target sequence from the *Hind*III site up to 200 bp in pGBDEL-5, or 180 bp in pGBDEL-9 and 140 bp in pGBDEL-11, upstream the unique *Xho*I site within the *glaA* promoter sequence;
- a 2 kb sized 3'-*glaA* target sequence from the *glaA* stop codon to *Sal*II site;
- the *amdS* gene as selectable marker under the control of *gpdA* promoter;
- two 1 kb sized 3'-*glaA* DNA direct repeats to facilitate later on the removal of the *amdS* selectable marker gene and,
- an additional restriction site (*Bam*HI in pGBDEL-5; *Sal*II in pGBDEL-9 and *Bgl*II in pGBDEL-11) positioned at the *glaA* truncation.

25

30 By using these pGBDEL replacement vectors, 4.3 kb *glaA* sequences in the *A. niger* host which will become deleted, which includes the 2 kb sized *glaA* promoter sequences (ranging from 200, 180 bp or 140 bp in pGBDEL-5, pGBDEL-9 and pGBDEL-11, respectively, upstream the *Xho*I) up to the ATG start codon and the entire *glaA* encoding sequence. For a schematic view figure 2).

35 **1.2.c. Construction of pGBGLA intermediate vectors.** For the construction of the intermediate vector pGBGLA16 two oligo nucleotides (30 / 31) were synthesized containing the enzyme restriction sites: *Not*I, *Eco*RI, *Kpn*I, and *Xho*I. Oligo were adapted with a *Hind*III restriction site at 5'-

end and with an *Eco*RI restriction site at 3'-end. By insertion of this oligo nucleotide into pTZ18R the *Eco*RI site will be destroyed.

Both oligo nucleotides 30 and 31 were cloned into the *Eco*RI 5 and *Hind*III sites of plasmid pTZ18R. Control digests were performed to be certain whether the desired enzyme restriction sites for *Kpn*I, *Xho*I, *Not*I and *Hind*III were incorporated appropriately and the *Eco*RI site destroyed. Derived plasmid was designated pTM1 (see figure 3)

10 From pTM1, the *Eco*RI and *Kpn*I DNA fragment was isolated and purified by gel electrophoresis after digestion with both restriction enzymes, to insert the *Eco*RI/*Kpn*I fragment of pGBDEL4L comprising the *P_{gpdA}/amdS* sequence. The construction of pGBDEL4L is described extensively in our previous Patent 15 Application (EPA 0635574A1). pGBDEL4L was digested with *Eco*RI/*Kpn*I and *Xho*I (the latter one to avoid molecular cloning of another DNA fragment of pGBDEL4L possessing the same size). The correct *Eco*RI/*Kpn*I DNA fragment, comprising the *P_{gpdA}/amdS* sequence, was purified by gel 20 electrophoresis and cloned into the *Eco*RI/*Kpn*I sites of pTM1. This intermediate vector was designated pGBGLA16 (see figure 3).

25 To insert the 2.2 kb sized 3'-*glaA* sequence appropriately into the pGBGLA16, the fragment needs some modifications first. A fusion PCR was performed with pAB6-1 as template and two sets of oligo nucleotides as primers (32/33 and 34/35), to destroy the *Kpn*I restriction site within 2.2 kb 30 3'-*glaA* sequence, and to create appropriate cloning sites at the 5'-boundary (*Kpn*I) and at the 3'-boundary (*Xho*I, *Hind*III). Template pAB6-1 comprises a 16 kb large *Hind*III fragment in pUC19, containing the *glaA* genomic locus (molecular cloning is extensively described in EPA 0357127A1).

35 In the first PCR, oligo nucleotides 32 and 33 were used as primer set to amplify a part (1 kb) of the 3'-*glaA* sequence. In the second PCR oligo nucleotides 34 and 35 were used to amplify the remaining 1.2 kb sequence of the

3'-glaA flanking.

After purification by gel electrophoresis both amplified fragments were used as template in a fusion PCR with oligo nucleotides 32 and 35 as primer set. A schematic presentation of the PCR fusion is presented in figure 4.

The obtained 2.2 kb 3'-glaA PCR fusion fragment was purified by gel electrophoresis, digested with *Kpn*I and *Xho*I and inserted by molecular cloning into the *Kpn*I/*Xho*I sites of pGBGLA16, yielding pGBGLA18 (see figure 4).

10

1.2.d. Construction of glaA replacement PGBDEL vectors. In obtaining the three final pGBDEL vectors, the 5'-glaA target sequence, an unique restriction site for each vector, and the 1 kb 3'-glaA sequence as direct repeat have to be inserted into the *Hind*III/*Not*I sites of pGBGLA18. To this end, three separate fusion PCR's were carried out to construct pGBDEL5, -9 and -11.

Construction of pGBDEL5.

20 A 1th PCR was carried out to modify the 5'-glaA target sequence at the 3'-border with two synthetic oligo nucleotides: 36, containing a part of the nucleotide sequence of pUC19, the *Hind*III site of the 5'-glaA sequence and 37 matching about 200 bp upstream the *Xho*I site in the glaA promoter, which comprises an additional *Bam*HI site, stop-codons in all reading frames and the first 18 nucleotides (5'-border) of the 1 kb 3'-glaA direct repeat. The 2nd PCR was performed to modify the 3'-glaA direct repeat at the 5'-border with oligo nucleotide 38, which is the reverse of 37 and oligo nucleotide 39, matching with sequences around the *Eco*RI site of the 3'-glaA direct repeat, added with the restriction sites *Xba*I and *Not*I for appropriate cloning into pTZ18R and pGBGLA18 respectively. In both PCR amplification pAB6-1 was used as template.

35 Amplified DNA fragments of both PCR's were separated by gel electrophoresis and used as templates in the fusion PCR with 36 and 39 as primers. The obtained 3 kb sized DNA fusion fragment was purified by gel electrophoresis,

digested with *Hind*III and *Not*I and subsequently molecular cloned into the appropriate sites into pGBGLA18, yielding the first gene replacement vector pGBDEL5 (see figure 5).

5 **Construction of pGBDEL9.**

The 1th PCR was carried out with oligo's 36 and 49 (as described above) matching about 180 bp upstream the *Xho*I site in the *glaA* promoter, which comprises an additional *Sal*II site, stop-codons in all reading frames and the first 10 18 nucleotides (5'-border) of the 1 kb 3'-*glaA* direct repeat. The 2nd PCR was performed to modify the 3'-*glaA* direct repeat at the 5'-border with oligo nucleotides 50, which is the reverse of 49 and oligo nucleotide 39.

In both PCR amplifications pAB6-1 was used as template. 15 Amplified DNA fragments of both PCR's were separated by gel electrophoresis and used as templates in the fusion PCR with oligo's 36 and 39 as primers. The obtained 3 kb sized DNA fusion fragment was purified by gel electrophoresis, digested with *Hind*III and *Not*I and subsequently molecular 20 cloned into the appropriate sites into pGBGLA18, yielding the second *glaA* gene replacement vector pGBDEL9 (see figure 6).

Construction of pGBDEL11.

25 The 1th PCR was carried out with oligo's 36 and 51 matching about 140 bp upstream the *Xho*I site in the *glaA* promoter, which comprises an additional *Bgl*III site, stop-codons in all reading frames and the first 18 nucleotides (5'-border) of the 1 kb 3'-*glaA* direct repeat. The 2nd PCR was performed 30 to modify the 3'-*glaA* direct repeat at the 5'-border with oligo nucleotides 52, which is the reverse of 51 and oligo nucleotide 39.

In both PCR amplification pAB6-1 was used as template. Amplified DNA fragments of both PCR's were separated by gel 35 electrophoresis and used as templates in the fusion PCR with 36 and 39 as primers. The obtained 3 kb sized DNA fusion fragment was purified by gel electrophoresis, digested with *Hind*III and *Not*I and subsequently molecular

cloned into the appropriate sites into pGBGLA18, yielding the third gene replacement vector pGBDEL11 (see figure 7).

1.2.e Deletion of *glaA* promoter and coding sequences and
5 incorporation of specific "DNA-flags" in *A. niger*. In this example the successful deletion of all three *glaA* genes present in *A. niger* 646.97 is described by using the pGBDEL vectors and the "MARKER-GENE FREE" technology in three successive transformation rounds.

10 By linearizing pGBDEL vectors with *Xba*I and *Hind*III and the fact that the rDNA cassettes are flanked by DNA sequences homologous to the *glaA* target locus of the host genome, pGBDEL vectors will become integrated by a double cross-over event at one of the *glaA* loci of the host, 15 resulting in a replacement of the *glaA* sequence by the truncated *glaA* loci of the pGBDEL vectors. However, the frequency of targeting of a pGBDEL vector by a double crossing-over event is limited and dependent on the number of the *glaA* genomic target loci of the host. So, although 20 linearized vectors are used comprising 2 kb sized *glaA* homologous regions at both flanks, still the majority of the generated transformants contain random integrated vectors.

However, the transformant possessing the desired genetic 25 feature could be easily selected and verified, by applying a) the PCR-based DNA-flag test, b) the target specific PCR-procedure and c) by detailed Southern analyses as indicated in Materials & Methods.

30 Finally, once the appropriate transformant was genetically verified the *amdS* marker gene was removed by applying the fluoroacetamide counter selection procedure. Due to the incorporated 3-*glaA* DNA repeats, flanking the P_{gpdA} /*amdS* marker gene cassette, after each round the *amdS* selection 35 marker was eliminated by an internal recombination event. Thus obtained modified *A. niger* strains were subjected to successive transformation rounds to eliminate and modify the remaining *glaA* loci.

Modification of the first *glaA* amplicon in *A. niger* CBS 646.97 with pGBDEL5.

A. niger CBS 646.97 was transformed with 5 µg linearized (HindIII/XhoI) pGBDEL5 DNA. Transformants were selected on selective plates containing acetamide and spores prepared after repeated growth on selective medium.

A restricted number of transformants were selected and isolated chromosomal DNA extensively analysed by Southern 10 analyses using *Kpn*I and *Bam*HI digests and the 5'-*glaA* HindIII/XhoI and the 2.2 kb *Sal*I 3'-*glaA* fragments as probes. See figure 8 for comparising the *Bam*HI hybridization patterns of the Δ *glaA* loci of the host strain and the two remaining Δ *glaA* loci of the transformant.

15 Transformants showing the expected hybridization patterns as indicated in figure 9 were selected.

Subsequently, on these transformants the counter slection procedure was performed. Spores were seeded on fluoroacetamide containing medium (for details see 20 Materials & Methods). On average 1-2% of the seeded spores were able to grow on these selective conditions. Cassette PCR analyses, performed directly on mycelium of these growing colonies with *amdS* specific oligo's 15 and 16 as primers , revealed that all growing cells are indeed 25 recombinant cells, which have lost the *amdS* marker gene.

Surprisingly, however, detailed Southern analysis on many 30 recombinant cells showed that in most cases (frequency about 90%) hybridization patterns were observed which are characteristic only for the parental strain 646.97, instead of the expected pattern for one truncated *glaA* locus and the two remaining intact *glaA* loci. In addition, the intensities of hybridizing DNA fragments pointed out that the number of *glaA* amplicons was not diminished in these 35 *amdS*-negative strains. Approximately 10% of the cells show the expected hybridization pattern as indicated in figure 10. So, in those cases the *amdS* marker-gene was indeed deleted via the internal recombination event over the flanking 3'-*glaA* direct repeats, as expected.

This unexpected recombination event in most of the *amdS* negative strains can be explained only by the occurrence of a genetic phenomenon, called gene-conversion. A feature 5 which is used later on (see example 1.5 and 1.6) to amplify enzyme encoding expression cassettes targeted at one of the truncated *glaA* amplicons of the *A. niger* CBS 646.97 host.

One *amdS*-negative strain, showing the hybridization 10 patterns for one truncated *glaA* locus and two intact *glaA* loci as presented in figure 10, was designated as *A. niger* GBA-201 and subjected to a second transformation with pGBDEL-9.

15 **Modification of the second *glaA* amplicon in *A. niger* CBS 646.97 with pGBDEL9.**

A. niger GBA-201 was transformed with 5 µg linearized (*Hind*III/*Xba*I) pGBDEL9 DNA. Again transformants were selected on selective plates containing acetamide and 20 spores prepared after repeated growth on selective medium. Because targeting of pGBDEL9 can occur also on the previous "BamHI-marked *glaA* truncated locus of the host, only those transformants were selected and analysed by Southern analyses, showing still the presence of that truncated 25 "BamHI" *glaA* locus. To this end, first the PCR-based "DNA-flag" test was performed on mycelium of these transformants. Only those transformants showing the 200 bp DNA fragment characteristic for the "BamHI" truncated *glaA* locus were analyzed by the PCR-methods, followed by an 30 extensive Southern analyses as indicated above.

Subsequently, spores of the transformant showing the correct hybridization pattern (figure 10) were seeded on fluoroacetamide containing medium (for details see Materials & Methods). Again on average 1-2% of the seeded 35 spores were able to grow on these selective conditions. All of them appeared to have lost the *amdS* gene as revealed after applying the cassette PCR-test on mycelium of these colonies.

As second selection criterium the "DNA-flag" test was performed. Only those transformants were subjected to detailed Southern analyses as described as above, showing the desired DNA-flag pattern (2 bands of 200 and 220 bp) 5 each characteristic for the presence of the "BamHI" truncated *glaA* amplicon and the "SalI" truncated *glaA* amplicon, respectively.

One *amdS*-negative strain, showing the hybridization patterns for two truncated *glaA* amplicons and one intact 10 *glaA* amplicon (figure 12), was designated *A. niger* GBA-202 and subjected to a third transformation with pGBDEL-11.

Modification of the third *glaA* amplicon in *A. niger* CBS 646.97 with pGBDEL11.

15 *A. niger* GBA-202 was transformed with 5 µg linearized (*Hind*III/*Xba*I) pGBDEL-11 DNA. Again transformants were selected on selective plates containing acetamide and spores prepared after repeated growth on selective medium. Also in this case targeting of pGBDEL11 can occur on one of 20 the previous *glaA* truncated amplicons of the host. By performing the "DNA-flag" test, only those transformants were selected and analysed by Southern analyses, which still show the presence of the 200 and 220 bp fragments, representing the two previously truncated "BamHI" and 25 "SalI" *glaA* amplicons.

Transformant showing the correct hybridization patterns as indicated in figure 13 were subjected to the counter selection procedure to obtain marker-gene free recombinants. Again on average 1-2% of the seeded spores were 30 able to grow on these selective conditions. All of them appeared to have lost the $P_{gpdA}/amdS$ marker gene cassette as revealed after applying the *amdS* cassette PCR-test on mycelium of these colonies.

As second selection criterium the "DNA-flag" test was 35 performed. Only those transformants were subjected to detailed Southern analyses as described above, showing the desired DNA-flag pattern of 3 bands (200, 220 and 300 bp) referring to the presence of the "BamHI" as well as to

"SalI" and "BglII" truncated *glaA* amplicons, respectively. One *amdS*-negative strain, showing the hybridization patterns (figure 14) for all three truncated *glaA* amplicons, was designated as *A. niger* ISO-505.

5

1.3. Description and construction of pGBAAS-1 and pGBTOPFYT-1 expression vectors.

10 1.3.a **Rationale.** pGBTOP vectors, comprising the expression cassette for an enzyme, are introduced into the *A. niger* host strains by co-transformation with an *amdS* selectable marker-gene containing vector, which is designated as pGBAAS-1. Both vectors comprise two DNA domains homologous 15 to the *glaA* locus of *A. niger* host strain to direct targeting of the linearized plasmid to one of the truncated *glaA* loci of *A. niger* ISO-505. These domains, each approximately 2 kb in size, are homologous to the downstream non-coding *glaA* sequence, and are specified as 20 3'- and 3''-*glaA* domains. Between these domains a unique restriction site is incorporated (*Xba*I in pGBAAS-1 and *Hind*III in pGBTOPFYT-1) to obtain, after removal of the *E.coli* pTZ18R cloning vector prior to transformation by digestion, DNA enzyme restriction ends which are almost 25 complementary to the *glaA* target locus of the host.

In pGBAAS-1 the *amdS* gene is driven by the strong *gpdA* promoter. By using such a promoter the majority of obtained 30 transformants will possess just a single integrated selection-marker cassette. Having such transformants are crucial for performing the next step: selecting for MARKER-GENE FREE recombinants strains by applying the previously described MARKER-GENE FREE technology. The presence of 35 multiple *amdS* genes, will certainly affect the frequency to remove them in the "one step counter selection procedure" or will be even impossible.

In pGBTOPFYT-1 *phyA* is driven by *PglaA*. As indicated above

already, the composition of this vector is designed in such a way that linearized expression cassette become integrated at one of the *glaA* target loci in *A. niger* ISO-505. To get transformants possessing multiple copies of the expression 5 cassette versus a single copy of the co-vector (necessary to obtain finally MARKER-GENE FREE recombinant strains) in just one transformation round and both targeted to the same *glaA* target locus of the host genome, the ratio of linearized DNA fragments of both cassettes is crucial.

10

3.1.b. Construction of the integration vector pGBAAS-1. All details concerning the construction of pGBAAS-1 can be found in one of our previous patent applications EPA 0635574A1. In this patent application the construction of 15 the *amdS* selection marker-gene vector pGBGLA-50 is described extensively. For nomenclature reasons only, later on this vector is renamed as pGBAAS-1(*Aspergillus AmdS Shuttle*). See figure 15 for the physical map.

20 **3.1.c. Construction of the integration vector pGBTOPFYT-1.** Also the construction of pGBTOPFYT-1 can be found in the same patent applications as mentioned above. In that patent application the construction of the phytase expression vector pGBGLA-53 is described extensively. Again for 25 nomenclature reasons only, this expression vector was later on renamed as pGBTOPFYT-1 (Tool to Over express Proteins). See figure 16 for the physical map.

1.4 Development of a MARKER-GENE FREE, phytase producing *A. niger* strain, containing one or multiple phytase expression cassettes all targeted at one of the truncated *glaA* amplicons of *A. niger* ISO-505.

5 1.4.a. **Rationale.** The aim of this example is to show that expression cassettes (just one or even multiple copies) can be directed perfectly to a predefined target locus in the genome of a host cell by co-transformation with a vector possessing a selectable marker gene. The second part shows
10 how to remove the selectable marker gene cassette by an internal recombination event without loosing the targeted (multiple) enzyme expression cassette(s).

The target locus of choice in this case is the 4 kb *glaA* sequence located just downstream the *glaA* stop codon.
15 Integration of both linearized plasmids pGBTOPFYT-1 and pGBAAS-1 occurs via a single cross-over event at one of the truncated and marked *glaA* loci of the *A. niger* ISO-505 strain.

20 To obtain finally selection MARKER-GENE FREE recombinants, only those transformants will be selected for the subsequent fluoroacetamide counter selection procedure, possessing both plasmids targeted in an appropriate way at the same marked *glaA* locus of the host genome.

25 1.4.b **Co-transformation of *A. niger* ISO-505 with linearized pGBAAS-1 and pGBTOPFYT-1 DNA.** Both plasmids, pGBAAS-1 and pGBTOPFYT-1, were linearized by digestion with *Xba*I and *Hind*III, respectively. The 2.8 kb *E.coli* cloning sequence was removed prior to transformation by gel electrophoresis.
30 A ratio of 1 to 5 µg linearized DNA of pGBAAS-1 and pGBTOPFYT-1, respectively, was used to transform *A. niger* ISO-505 as described in Materials & Methods. Transformants were selected on acetamide plates. Spores of these transformants were isolated by plating the individual
35 colonies on PDA-plates. Approximately 500 transformants were subjected to the cassette PCR-test.

1.4.c Selection for co-transformants. Transformants possessing the *amdS* marker gene as well as the *phyA* expression cassette were identified by the cassette PCR test using phytase specific oligo nucleotides 42/43 as 5 primers. Positive transformants containing one or multiple *phyA* expression cassette(s) will show a specific DNA band of 482 bp in size. Co-transformation frequencies varied between 10 to 50%.

10

1.4.d Selection of targeted *amdS*⁺, *phyA*⁺ co-transformants. On identified co-transformants, targeting PCR tests were performed using two sets of oligo nucleotides 46/47 (primer set 1) and 46/48 (primer set 2). Co-transformants showing a 15 positive result (amplification of a 4.2 kb DNA fragment, see figure 17) with one these two primer sets were selected. A negative result indicates that all cassettes are integrated randomly, a positive result with primer set 1 or 2, implies that either the *phyA* or *amdS* is located 20 adjacent to the *glaA* target locus, respectively. Between 5-50% of the co-transformants appeared to be positive. A limited number of identified strains were selected for extensive Southern-analyses.

25 1.4.e Genetic analyses of targeted *amdS* and *phyA* targeted co-transformants by Southern analyses. Chromosomal DNA was isolated and Southern analyses performed using *Bgl*II digests hybridized with the *amdS* and the 2.2 kb *Sal*I/*Xba*I 3"-*glaA* DNA fragment as probe. Schematic drawings 30 of six mostly observed DNA hybridization patterns are shown in figures 18 to 23. Co-transformants showing the hybridization pattern characteristic for possessing a single *amdS* copy and one or multiple *phyA* cassette(s) (figures 20-23). were subjected to fluoroacetamide counter- 35 selection procedure to remove finally the *amdS* selection marker gene.

1.4.f Selection of *amdS* gene free (*amdS*⁻) phytase producing

strains. Recombination over the direct repeats, created by targeted integration of the *amdS* expression cassette, will result in the loss of the *amdS* cassette and can be selected on fluoroacetamide medium. Here we describe the selection 5 of *amdS* free recombinant strains, which still contain the adjacent (multiple) integrated *phyA* expression cassette(s) by using the Fluoroacetamide counter selection procedure.

Spores of selected targeted *amdS*⁺/*phyA*⁺ co-transformants were plated onto fluoroacetamide plates. Initially, growing 10 colonies were analyzed by several successive PCR-based tests. First, all progenies were tested for the genotype, *amdS*⁻. The selection for *amdS*⁻ genotypes were done by performing the cassette PCR test on colonies using the *amdS* specific primers as described in Material & Methods. 15 Surprisingly, however, in the subsequent PCR-based "DNA-flag" test, it appeared that in the majority of the progenies an entire *glaA* amplicon was lost as well. In addition, in most *amdS*⁻ recombinants the targeted *phyA* cassettes were lost as well, as appeared in the third PCR- 20 test using the *phyA* specific primer set.

These results can be explained only by the occurrence of one phenomenon, which is known as gene-conversion. In several cases a rather large DNA fragment or even the size of the entire *glaA* amplicon (≥ 80 kb) was replaced by 25 another *glaA* amplicon. In most progenies (over 90%), however, it appeared that an entire *glaA* amplicon was deleted. Hence, to cover observed results entirely, terms as amplicon conversion and deletion are more appropriate.

Observed phenomenon was used to determine whether all *amdS* 30 and *phyA* cassettes in co-transformants are targeted in the same truncated *glaA* locus and in which one of them. To determine this, just one or two fluoroacetamide resistant colonies of each co-transformant was tested which of them will result in the genotype: *amdS*⁻/*phyA*⁻/*glaA*-amplicon²⁺ by 35 performing a mixed PCR-test using *phyA* and "DNA-flag" oligo nucleotides primer sets. Results showed that co-transformants can be realized possessing *amdS* and *phyA* cassettes integrated in one of the three *glaA* amplicons.

Subsequently, from such identified co-transformants, additional progenies were tested by applying a mixed PCR test with the *phyA* and "DNA-flag" primer sets. The 5 occurrence of the desired *amdS*⁻/*phyA*⁺/*glaA*-amplicon³⁺ genotype varied strongly (1 to 20%) among the progenies of the individual co-transformants. This frequency appeared to be strongly dependent on the genetic composition of the *amdS* and *phyA* cassettes within the *glaA* amplicon of the 10 pre-selected co-transformants.

In case, in a co-transformant multiple cassettes have become targeted at one *glaA* locus many repeats are created. The consequence for the fluoroacetamide counter selection procedure is that loss of the *amdS* cassette can occur 15 differently, resulting in *amdS*⁻ progenies possessing a different number of *phyA* cassettes.

For this reason, of each pre-selected co-transformant, several progeny recombinants were selected (all showing the desired *amdS*⁻/*phyA*⁺/*glaA* amplicon³⁺ genotype) but containing 20 different number of remaining *phyA* cassettes.

Pre-selection for such progeny strains could be easily done by comparing the difference in intensity of the amplified *phyA* specific DNA bands to the DNA bands representing the three *glaA* amplicons in the above described PCR test.

25 Finally, to confirm the genetic composition of selected *amdS* progenies, Southern analyses were done on chromosomal DNA digested with *Bam*HI and *Bgl*III, hybridized with a 2.2 kb *Sal*I/*Xho*I 3'-*glaA* DNA fragment as specific probe. Strains 30 showing the hybridization pattern as indicated in figure 24 for having 1, 2 or 3 *phyA* cassettes targeted in one the *Bam*HI-marked *glaA* amplicon, were selected and designated as *A. niger* NP505-1, -2 and -3, respectively.

These strains, together with those which possess the 35 phytase cassettes in one of the two other *glaA* amplicons, were subsequently tested for their ability to produce phytase.

Shake flask fermentations were carried out as indicated in

Materials & Methods. Measurement of the phytase activity in the supernatant revealed that all strains produce the same amount of phytase (100 U/ml) per *phyA* gene copy and appeared to be independent of the *glaA* amplicon in which 5 the *phyA* genes are localized.

1.5 Selection of convertants comprising an increase in phytase cassette from 1 to 2.

10 **1.5.a Rationale.** The copy-number of an expression cassette targeted at a DNA amplicon can be increased through gene conversion. Consequently, production of the enzyme encoded by the gene can increase. Here we describe the selection and characterization of *phyA* convertants, showing an 15 enhanced production of phytase.

1.5.b Selection of putative *phyA* convertants. As an example, strain *A. niger* NP505-2 was chosen. Recombinant strains containing less or more *phyA* cassettes targeted at 20 another *glaA* amplicon can be used as well. Spores of *A. niger* strain NP505-2, containing two *phyA* cassettes targeted at the *Bam*HI-marked *glaA* amplicon were seeded onto plates containing PDA medium and after 2-3 days the DNA-flag PCR test was performed on individual colonies using 25 the *phyA* DNA-flag specific primers (see Materials & Methods). Using this method a genetic exchange (conversion/amplification or deletion) between the three marked *glaA*-amplicons can be visualized quite rapidly. Although the frequencies of obtained genotypes vary strongly, on 30 average we observed that over 95% of the tested colonies show no change at all of all three *glaA* amplicons. 5% of the progenies showed either a deletion of one or two *glaA* amplicons, or an amplification of one of the *glaA*-amplicons and the desired convertant was detected as well, as result 35 of a conversion between the "Bam"HI-marked" *glaA* locus and one of the other two *glaA* amplicons (an example of each is presented in figure 25).

1.5.c **Southern analyses of *phyA* convertants.** To determine whether the copy number of *phyA* in these convertants are doubled as well, several of such convertants (genotype *Bam*HI²⁺/*Sal*I⁺/*Bgl*III⁻ or *Bam*HI²⁺/*Sal*I⁻/*Bgl*III⁺) were subjected 5 to extensive Southern analyses using *Bam*HI and *Bgl*III digests probed with the *glaA* specific *Hind*III/*Xho*I 5'-*glaA* DNA fragment and the *Sal*I/*Xho*I 3"-*glaA* DNA fragment, respectively.

Hybridization patterns of *Bam*HI digests of the parental 10 strain and selected convertants are showed in figure 25. As expected, and compared to the parental *A. niger* NP505-2 strain, the PCR identified *Bam*HI²⁺ genotypes showed indeed in *Bam*HI digests reversed intensities for the 2.5 kb and 4.2 kb hybridizing DNA fragments. In the *Bam*HI²⁺ genotype 15 the 2.5 kb fragment represents two *Bam*HI-marked *glaA* amplicons and the 4.2 kb fragment the remaining *Sal*I or *Bgl*III *glaA* amplicon, depending on the genotype of selected *Bam*HI²⁺ convertants. Observed results clearly indicate that a predefined amplicon can be multiplied by "gene 20 conversion" at the cost of another related amplicon.

Hybridization patterns of *Bgl*III digests clearly showed that in selected *Bam*HI²⁺ convertants the number of *phyA* gene copies are doubled as well. As indicated in figure 26, a 25 *Bgl*III digest of chromosomal DNA of the parental strain show 5 hybridizing DNA fragments using the 3"-*glaA* fragment as probe. The 5 bands of 14.9, 11.7, 7.5, 5.7 and 5.6 represent, respectively, the 5'-flanking *glaA* sequence, the *Sal*I-marked amplicon, the *phyA*-*phyA* transition, the 30 3'-flanking *glaA* sequence and the *Bgl*III-marked *glaA* amplicon. In the parental strain al these bands have more or less the same hybridizing intensity. As shown in figure 26 as well, in selected convertants with genotype *Bam*HI²⁺/*Sal*I⁺/*Bgl*III⁻ or *Bam*HI²⁺/*Sal*I⁻/*Bgl*III⁺, the 5.6 kb or the 35 11.7 kb DNA fragment, respectively, is absent while the intensity of the 14.9, 7.5 and 5.7 kb hybridizing fragments is doubled.

This result indicates that the 2 *phyA* cassettes located within the *Bam*HI-marked *glaA* amplicon of the parental

strain are doubled as well by the conversion event in selected *Bam*HI²⁺convertants.

5 **1.5.d Analyses of *phyA* convertants for the production of phytase.**

To determine whether selected *phyA* convertants show indeed an increased production of phytase, shake flask fermentations were carried out as described in Materials & Methods.

10 On average the phytase expression of the parental strain NP505-2 (possessing 2 *phyA* gene copies) was measured at a level of about 200 U/ml. As predicted for both selected convertants with genotype *Bam*HI²⁺/*Sal*I⁺ or *Bam*HI²⁺/*Bgl*II⁺, phytase levels up to 400 U/ml could be detected.

15 Both strains, designated as *A. niger* NP505-4 and -5, were used in example 1.6 to increase the *phyA* gene copy number furthermore.

20 **1.6 Selection of convertants comprising multiple modified DNA amplicons.**

1.6.a **Rationale.** Expression cassettes targeted in one of the DNA amplicons present in a host strain can be multiplied just by screening progenies of the parental strain and selecting for those recombinant strains in which the desired conversion had occurred. Although to low extent, as described above, conversions among these DNA amplicons occur spontaneously. This process of gene conversion can be used repeatedly, resulting in new convertants which possess an enhanced number of (*phyA*) modified DNA amplicons. By using this approach, we have identified a convertant possessing finally 6 *phyA* gene copies, all equally divided over the three modified *glaA* amplicons of the *A. niger* ISO-505 host.

35 The degree of "the enrichment of enzyme expression cassette" is dependent on the number of DNA amplicons originally present in the host strain. However, by applying this PCR-based DNA-flag test it appeared to be possible as

well, to isolate progenies having a DNA-flag profile which could be explained only by a spontaneous amplification of one of three *glaA* DNA amplicons. Using this repeatedly, recombinant strains could be selected finally, possessing 5 additional *glaA*-DNA amplicons compared to the host strain, each containing 2 *phyA* expression cassettes.

1.6.b. Selection of putative convertants comprising 6 *phyA* gene copies. Spores of *A. niger* strain NP505-4, 10 described in example 1.5.(d), containing 4 *phyA* expression cassettes in total (two *phyA* cassettes in each *Bam*HI-marked *glaA* amplicon) were seeded onto PDA plates and after 2-3 days the DNA-flag PCR test was performed on the individual 15 progenies using the DNA-flag specific primers. As stated before, again approximately 95% of the tested colonies show no change in *glaA* amplicon genotype. Approximately 5% of the progenies showed a DNA-flag pattern directing either to a deletion of one of the *Bam*HI- or the *Sal*I-marked 20 amplicons or even to a spontaneous amplification of one of the *Bam*HI-marked *glaA*-amplicons. Besides these recombinant strains, convertants having the desired DNA-flag pattern, the 200 bp sized *Bam*HI DNA-flag band with a slightly increased intensity, was identified as well. All progeny 25 strains showing the genotype "*Bam*HI³⁺" and "*Bam*HI³⁺/*Sal*I⁺" were subjected to Southern analyses.

To increase the *phyA* gene in the "*Bam*HI³⁺/*Sal*I⁺" convertant up to 8, spores were seeded onto PDA plates and after 2-3 days of growth the DNA-flag PCR test was performed on the individual progenies using the DNA-flag specific primers. 30 At a rather low frequency (1 out of 1000) a "*Bam*HI⁴⁺" convertant showing a DNA-flag pattern with an increased intensity of the 200 bp sized *Bam*HI-flag DNA fragment and the subsequent loss of the remaining 220 bp sized *Sal*I-flag DNA fragment (figure 27). This convertant strain was 35 subjected to Southern analyses as well.

1.6.c Genetic characterization of convertants by Southern analyses.

The *phyA* gene copy number of above identified convertants (PCR-based genotypes "*Bam*HI³⁺", "*Bam*HI³⁺/*Sal*I⁺" and "*Bam*HI⁴⁺" was determined by Southern analyses using *Bam*HI and *Bgl*II digests, hybridized respectively with *Hind*III/*Xba*I 5'-*glaA* 5 and *Sal*I/*Xba*I 3"-*glaA* specific DNA fragments, as probes.

Expected hybridization patterns of the *Bam*HI digests of the parental convertants (genotype *Bam*HI²⁺/*Sal*I⁺/*Bgl*II⁻ or *Bam*HI²⁺/*Sal*I⁻/*Bgl*II⁺) and selected convertants thereof are shown in figure 25 and 27. *Bam*HI digests show indeed a sole 10 hybridizing fragment of 2.5 kb for the "*Bam*HI³⁺" convertant. For the "*Bam*HI³⁺/*Sal*I⁺" an additional band of 4.2 kb was detected, indicating that in this convertant the *Sal*I-marked amplicon is still present as predicted on the result obtained from the DNA-flag pattern. Convertant 15 "*Bam*HI⁴⁺" shows the same hybridization pattern as the "*Bam*HI³⁺" convertant. For comparison the hybridization patterns of the original parental strain, *A. niger* NP505-2 and the two parental convertant strains *Bam*HI²⁺/*Sal*I⁺/*Bgl*II⁻ and *Bam*HI²⁺/*Sal*I⁻/*Bgl*II⁺, see previous figures.

20 Expected hybridization patterns of a *Bgl*II digest on DNA isolated from the above mentioned strains are indicated in figure 28. For the "*Bam*HI³⁺" as well as for the "*Bam*HI⁴⁺" convertant three hybridizing bands (14.5, 7.5 and 5.7) were 25 detectable, all with equal intensities, indicating that besides the 5'- and 3'-flanking sequences the *phyA* gene copies are increased up to 6 and 8, respectively. For convertant "*Bam*HI³⁺/*Sal*I⁺" the expected pattern was observed, including the 11.7 kb sized band (characteristic 30 for the presence of the *Sal*I amplicon). The intensity of this band is of course three-fold less.

These results clearly indicate that convertants can be selected, containing three and even four *Bam*HI-marked *glaA* 35 amplicons, all containing 2 *phyA* cassettes.

5 fermentations. To determine whether selected "BamHI³⁺" and "BamHI⁴⁺" convertants show indeed an increased production of phytase, shake flask fermentations were carried out as described in Materials & Methods. On average the phytase expression of the parental strain NP505-2 (possessing 2 phyA gene copies) was measured at a level of about 200 U/ml. As predicted for these three selected convertants phytase levels were measured of approximately 600 U for the "BamHI3+/SalI⁺" and "BamHI³⁺" genotypes and approximately 10 800 U/ml for the "BamHI⁴⁺" convertant strain.

Later on these strains were designated as *A. niger* NP505-6, -7 and -8, respectively.

15 1.7 Direct selection of recombinants possessing enhanced phytase production levels

1.7.a Rationale. Due to the fact that the *glaA* DNA amplicons are marked by the so-called DNA-flags, convertants could be selected just by performing the PCR-based DNA-flag test. By applying this genetically-based screening approach we were able to show and to prove that the *phyA* expression cassettes were multiplied as a consequence of conversions among the *phyA* modified *BamHI*-marked *glaA* amplicon and the other two amplicons.

25 As stated above we observed a linear relationship between *phyA* gene copy numbers and phytase production levels, indicating that selection can be based as well on expression. In this example we show the isolation of convertants possessing increased *phyA* gene copy numbers by 30 screening progenies of an initial *BamHI-glaA* targeted *phyA* recombinant strain for enhanced phytase expression levels in micro-well plates.

1.7.b Screening for enhanced phytase expression levels.

35 Spores of single colonies of strain *A. niger* NP505-2 were inoculated in a 96-well plates, containing 200 µl *A. niger* fermentation medium as described in Materials & Methods. After 7 days of growth at 34 °C, 100% humidity and slightly

shaking, the phytase enzyme activity was determined in the supernatant of each well. Enzyme expression levels of approximately thousand progenies were tested. Cells showing an increased phytase activity were harvested, 5 spores collected and applied to an additional micro-titer screening round. Again, cells with increased phytase enzyme activity were found.

In subsequent shake flask fermentations such identified strains show phytase expression levels up to 600 U/ml.

10

1.7.c Genetic characterization of identified recombinants strains. Observed phytase production improvements of above described strains appeared to be the result of an increase of the *phyA* gene copy number, either by amplification or 15 conversion of the *phyA* containing *Bam*HI-marked *glaA* locus. Firstly, the genotype of these selected strains was determined by applying the DNA-flag test and secondly by Southern analyses.

The DNA-flag test clearly indicated that all sorts 20 "DNA-flag" genotypes were amongst the selected strains. These strains showed the same genotypes which have been isolated before already by performing the DNA-flag test as a selection criterium, like:

*Bam*HI²⁺/*Sal*⁺/*Bgl*III⁻, *Bam*HI²⁺/*Sal*⁺/*Bgl*III⁻,
25 *Bam*HI³⁺/*Sal*⁺/*Bgl*III⁻, *Bam*HI²⁺/*Sal*⁻/*Bgl*III⁺,
*Bam*HI³⁺/*Sal*⁻/*Bgl*III⁻.

All these genotypes were confirmed by Southern analyses as described in previous examples.

EXAMPLES *PENICILLIUM*5 **2.1 Selection and characterization of the *P. chrysogenum* host**

2.1.a **Rationale.** Amplification of inserted genes through gene conversion requires the presence of multiple homologous DNA domains in the genome. Such domains have been described for *P. chrysogenum* (in this report referred 10 to as PEN amplicons: Fierro et al., 1995 Proc. Natl. Acad. Sci. USA 92:6200-6204). The penicillin cluster, a 15 kb domain encompassing the penicillin biosynthetic genes, *pcbAB*, *pcbC* and *penDE*, is located on these PEN amplicons (Fig.29) (Diez et al., 1990 J. Biol. Chem. 265: 16358-15 16365; Smith et al., 1990 EMBO J. 9: 741-747). Here we describe the selection and characterization of *P. chrysogenum* host strain CBS 649.97, containing multiple copies of the PEN amplicon.

20 **2.1.b Quantitation of PEN amplicons.** *P.chrysogenum* strain CBS 649.97 was obtained by classical strain improvement from strain Wis54-1255 (ATCC 28089), which contains a single PEN amplicon (Fierro et al., 1995 Proc. Natl. Acad. Sci. USA 92:6200-6204). The number of PEN amplicons in 25 strain CBS 649.97 was determined by Southern analyses. To this end, chromosomal DNA was digested with *Bst*XI and hybridized to HELE and *niaD* probes. A 7-fold higher HELE/*niaD* hybridization ratio was detected for CBS 649.97 compared to Wis54-1255 (Fig.30,42). Similarly, *Not*I-digested DNA separated by CHEF, was hybridized to HELE 30 probe (figure 42). A major hybridization signal, approximately 420 kb larger in CBS 649.97 than in Wis54-1544, was detected (Fig.31). These results indicated the presence of about 7 PEN amplicons in strain CBS 649.97.

35

2.2 Construction of expression vectors

2.2.a **Rationale.** Directed insertion (targeting) of rDNA

molecules into the genome occurs through homologous recombination. Therefore, rDNA cassettes should be flanked by DNA fragments homologous to the target site in the genome (Fig.32). Two targeting domains within the 5 penicillin cluster, defined as HELE and HELF, were chosen for directed insertion of the expression cassettes into the PEN amplicons (Fig.29). Here we describe the design and construction of *amdS*, *cefE* and *cefF* expression vectors used in this report.

10

2.2.b Basic design of expression vectors. Linear DNA molecules are crucial for targeted integration into the genome. Furthermore, both 5' and 3' ends (flankings) must consist of DNA homologous to the desired integration site. 15 Transformation fragments, therefore, comprise the expression cassette (the gene of interest regulated by a suitable promoter and terminator) flanked by the 5' and 3' targeting domains. These fragments are cloned into an *E.coli* vector for propagation of the plasmid. The resulting 20 expression vectors are designed such that *E.coli* sequences are removed during linearization and isolation of the transformation fragment (Fig.32). Hence, recombinant strains will be free of *E. coli* DNA (E.P. 0 635 574 A1).

25 **2.2.c Construction of expression vector pHELE-A1.** The 5' HELE targeting domain was PCR-amplified from chromosomal DNA of *P. chrysogenum* strain CBS 649.97, using oligo's 1 and 2. The resulting product was cloned as a *NotI-SpHI* fragment in pZErOTM-1 (Invitrogen, Carlsbad, USA), yielding 30 plasmid pHELE5'. Similarly, the 3' HELE targeting domain was PCR-amplified using oligo's 3 and 4 and cloned as a *HindIII-NotI* fragment in pHELE5', yielding plasmid pHELF53. The *amdS* expression cassette, encompassing *amdS* regulated by *P_{gpdA}* and *T_{amdS}*, was PCR-amplified from plasmid pGBDEL4L 35 (E.P. 0 635 547 A1), using oligo's 5 and 6. The resulting product was cloned as a *NotI* fragment in pHELE53, yielding expression vector pHELE-A1 (Fig.33).

2.2.d Construction of expression vector pHELP-A1. The 5' HELF targeting domain was PCR-amplified from chromosomal DNA of *P. chrysogenum* strain CBS 649.97, using oligo's 7 and 8. The resulting product was cloned as a *NotI-XbaI* fragment in pZErO™-1 (Invitrogen, Carlsbad, USA), yielding plasmid pHELP5'. Similarly, 3' HELF flanking was PCR-amplified, using oligo's 9 and 10, and cloned as a *PstI-NotI* fragment in pHELP5', yielding plasmid pHELP53. The *amdS* expression cassette, encompassing *amdS* regulated by P_{gpdA} and T_{amdS} , was PCR-amplified from plasmid pGBDEL4L (E.P. 0 635 547 A1), using oligo's 5 and 6. The resulting product was cloned as a *NotI* fragment in pHELP53, yielding expression vector pHELP-A1 (Fig.34).

2.2.e Construction of expression vector pHELE-E1. The P_{pcbC} was PCR-amplified from genomic DNA of *P. chrysogenum* strain CBS 649.97, using oligo's 11 and 12. The resulting product was cloned as a *XhoI-NdeI* fragment in pGSEWA (WO 95/04148), yielding pISEWA-N. Finally, the *cefE* expression cassette, regulated by P_{pcbC} and T_{penDE} , was cloned as a *NotI* fragment from pISEWA-N in pHELE53, yielding expression vector pHELE-E1 (Fig.35).

2.2.f Construction of expression vector pHELP-F1. The *cefF* gene was PCR-amplified from genomic DNA of *S. clavuligerus* ATCC 27064, using oligo's 13 and 14, according to the Expand™ Long Template PCR System (Boehringer Mannheim). Cycle conditions: 30 x (1 min 98°C, 5 min 70°C), 1 x (7 min 72°C). The resulting product was cloned as a *NdeI-NsiI* fragment in pISEWA-N (substituting *cefE*), yielding pISFWA. Finally, the *cefF* expression cassette, regulated by P_{pcbC} and T_{penDE} , was cloned as a *NotI* fragment from pISFWA in pHELP53, yielding expression vector pHELP-F1 (Fig.36).

2.3 Modification of PEN amplicons with *cefE*
2.3.a Rationale. Efficient selection of transformants requires a selectable marker. Co-transformation of fungi is a well known procedure by which the host is transformed

simultaneously by two different rDNA molecules; one containing the gene of interest and the other the selection marker. Use of the bi-directional *amdS* marker allows repeated transformation of the *P. chrysogenum* host (E.P. 0 5 635 547 A1). Recombination over the direct repeats created by targeted integration, will result in loss of the *amdS* cassette and can be selected on Fluoroacetamide medium (Fig.37). Here we describe selection of *amdS*-free recombinants containing the *cefE* expression cassette 10 integrated in one of the PEN amplicons.

2.3.b Selection of targeted *amdS*, *cefE* co-transformants. *P. chrysogenum* strain CBS 649.97 was co-transformed with *Sfi*I-linearized HELE-A1 and HELE-E1 fragments (Fig.33,35). 15 Transformants containing *amdS* were selected on acetamide plates and tested for *cefE* by cassette PCR, using oligo's 17 and 18 (Fig.40a). Co-transformants with *amdS* or *cefE* integrated in HELE, were identified by targeting PCR, using oligo's 19, 20 and 21 (Fig.40.b,c). These strains were used 20 for isolation of *amdS*⁻ recombinants.

2.3.c Selection of *amdS*⁻ (marker-free) *cefE* recombinants. Spores of *amdS*, *cefE*, co-transformants described in 2.3.b, were plated onto Fluoroacetamide medium. Recombinants 25 retaining *cefE* were identified by the *E. coli* bioassay. Loss of *amdS* but presence of at least one *cefE* copy was confirmed by cassette PCR, using oligo's 15, 16, 17 and 18 (Fig.40.a,d). These strains were selected for Southern analyses.

30 2.3.d Southern analyses of *amdS*⁻ recombinants. Integration of *cefE* in HELE and the number of *cefE* copies in *amdS*⁻, *cefE*⁺ recombinants was analyzed by Southern and TAFE. To this end, chromosomal DNA of recombinants was digested with 35 *Nru*I and hybridized to *cefE* probe (Fig.38,42). Similar hybridizations were performed with *Hpa*I-digested DNA, separated by TAFE (Fig.39). Recombinant strains with a hybridization pattern expected for integration of *cefE* in

HELE, were selected for further experiments.

2.4 Modification of a second amplicon with *amdS*

5 **2.4.a Rationale.** Gene conversion essentially substitutes the genetic information from an acceptor DNA strand by a donor DNA strand. Both donor and acceptor strands can be marked to visualize this event. Strains as described in 2.3 already have one amplicon marked by *cefE* (in this case the donor strand). Here we describe the modification of an 10 acceptor strand by insertion of a single *amdS* cassette in a different PEN amplicon than *cefE* (Fig.43).

15 **2.4.b Targeting of *amdS* to a second PEN amplicon.** Strains as described in 2.3.d, containing *cefE* in one amplicon, were transformed with *SfiI*-linearized HELE-A1 fragment (Fig.33). Integration of *amdS* and targeting to HELE was tested by cassette and targeting PCR using oligo's 15, 16 and 19, 20, respectively (Fig.40c,d). Integrations of *amdS* adjacent to *cefE* or multiple *amdS* copies in a single HELE 20 locus were identified by targeting PCR using oligo's 20, 21, 22 and 23 (Fig.40e,f,g). The remaining strains, hence, containing a single *amdS* cassette integrated in a second amplicon, were selected for Southern analyses.

25 **2.4.c Southern analyses of *amdS* transformants.** Chromosomal DNA of transformants described in 2.4.b was digested with *HpaI* and hybridized to *amdS* probe (Fig.41,42). Strains with a hybridization pattern expected for correct integration of *amdS* in HELE, were selected for gene conversion 30 experiments.

2.5 Increase of production by gene conversion

2.5.a **Rationale.** The copy number of an integrated gene increases through gene conversion (Fig.43). Consequently, 35 production of the enzyme encoded by the gene or its catalytic activity can increase. Recombinant *P. chrysogenum* strains expressing *cefE*, produce adipoyl-7-ADCA when fermented under the proper conditions (Crawford et al.,

1995 BIO/Technol. 13:58-62). Here we describe the selection and characterization of *cefE* gene convertants and the resulting increase of adipoyl-7-ADCA production.

5 **2.5.b Selection of *cefE* gene convertants.** Spores of strains described in 2.5.a, containing *cefE* and *amdS* in different PEN amplicons, were plated on Fluoroacetamide medium to select *amdS*⁻ recombinants. The *amdS*⁻, *cefE*⁺ genotype of these recombinants was confirmed by cassette PCR using
10 oligo's 15, 16, 17, 18, 26 and 27 (Fig40a,d,h). Oligo's 26 and 27 were included to amplify a fragment of the unique *niaD* locus, which served as an internal reference for relative quantitation of the PCR products. Gene convertants, identified as strains which lost the *amdS*
15 marker but gained *cefE* copies, as judged by the increased *cefE/niaD* ratio compared to the parental strain, were selected for Southern analyses.

2.5.c Southern analyses of *cefE* gene convertants.
20 Chromosomal DNA of strains selected in 2.5.b, were digested with *Nru*I and hybridized to *cefE* and *niaD* probes. Strains with a hybridization pattern identical to the parental strain, but increased ratio of the *cefE/niaD* hybridization signals, were selected for adipoyl-7-ADCA fermentations
25 (Fig.48).

2.5.d Adipoyl-7-ADCA production of *cefE* gene convertants. Adipoyl-7-ADCA production of gene convertants selected in 2.7.b, was determined by shake flask fermentations. All
30 selected strains produced significantly more adipoyl-7-ADCA than the corresponding parental strains.

2.6 Simultaneous increase of different expression cassettes by gene conversion
35 **2.6.a Rationale.** Domains encompassing large segments of the amplicons, or even extending the borders of the amplicons, can participate in gene conversion. Hence, the copy number of different genes adjacent to each other can increase

simultaneously through gene conversion. Recombinant *P. chrysogenum* strains expressing *cefE* and *cefF*, produce adipoyl-7-ADAC, when fermented under the proper conditions (Crawford et al., 1995 BIO/Technol. 13:58-62). Here we 5 describe the simultaneous gene conversion of *cefE* and *cefF* expression cassettes, targeted to different loci on the same PEN amplicon (*cefE* + *cefF*), and the resulting increase of adipoyl-7-ADAC production.

10 2.6.b Selection of (*cefE* + *cefF*) transformants. Strains as described in 2.3.d, containing *cefE* integrated in HELE, were co-transformed with *Sfi*I- linearized HELF-A1 and HELF-F1 fragments (Fig.34,36). Transformants were selected on acetamide plates and tested by cassette PCR for *cefF* and 15 *amdS*, using oligo's 15, 16, 17 and 25 (Fig.40d,j). Integration of *amdS* or *cefE* in HELF was determined by targeting PCR using oligo's 20, 21 and 24 (Fig.40j,k). Chromosomal DNA of these strains was digested with *Not*I, blotted and hybridized to HELE probe (Fig.44). Strains 20 with a hybridizing fragment corresponding to integration of *cefF* or *amdS* in HELF on the PEN amplicon already containing *cefE*, were used for selection of marker free. (*amdS*-) recombinants.

25 2.6.c Selection of MARKER-GENE FREE (*cefE* + *cefF*) recombinants. Spores of strains described in 2.6.b were plated on Fluoroacetamide medium for selection of *amdS*- recombinants. Recombinants that lost *amdS* but retained *cefE* and *cefF*, were identified by cassette PCR, using oligo's 30 15, 16, 17, 18, and 25 (Fig.40a,d,j). Chromosomal DNA of these strains was digested with *Nru*I and hybridized to *cefF* probe (Fig.42,45). Strains with correct integration of *cefF* in HELF were used for selection of gene convertants.

35 2.6.d Selection of (*cefE*+*cefF*) gene convertants. Spores of strains described in 2.6.c were grown to single colonies and analyzed for the amount of *cefE*, *cefF* and *niaD* by cassette PCR, using oligo's 17, 18, 25, 26 and 27

(Fig.40a,h,i). Gene convertants were identified by simultaneously increased ratio's of *cefE/niaD* and *cefF/niaD*, compared to the parental strains. Increased copy numbers of *cefE* and *cefF* was confirmed by Southern analysis. To this end, chromosomal DNA was digested with *Nru*I and hybridized to *cefE* and *niaD* probes (Fig.46). Blots were stripped and subsequently hybridized to *cefF* and *niaD* probes (Fig.47). Strains with increased ratios of both *cefE/niaD* and *cefF/niaD* compared to the parental strains, 10 were selected for adipoyl-7-ADAC.

2.6.e Adipoyl-7-ADAC production of (*cefE+cefF*) gene convertants. Adipoyl-7-ADAC production of gene convertants selected in 2.6.d, was determined by shake flask 15 fermentations. All selected strains produced increased amounts of adipoyl-7-ADAC compared to the parental strains.

Applicant's or agent's Int'l reference number	C 1202 PCT	International application No.
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>43</u> , line <u>15-17</u>	
B. IDENTIFICATION OF DEPOSIT	
Name of depositary institution Centraalbureau voor Schimmelcultures	
Address of depositary institution (including postal code and country) Oosterstraat 1 P.O. Box 273 3740 AG BAARN Netherlands	
Date of deposit April 8, 1997	Accession Number CBS 649.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input checked="" type="checkbox"/>	
Applicant makes use of Rule 28(4) EPC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
EP	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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B. IDENTIFICATION OF DEPOSIT	
Name of depositary institution Centraalbureau voor Schimmelcultures	
Address of depositary institution (including postal code and country) Oosterstraat 1 P.O. Box 273 3740 AG BAARN Netherlands	
Date of deposit April 8, 1997	Accession Number CBS 646.97
C. ADDITIONAL INDICATIONS (leave blank if not applicable)	
Applicant makes use of Rule 28(4) EPC	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
EP	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. 'Accession Number of Deposit')	
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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

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10

(ii) TITLE OF INVENTION: Gene conversion as a tool for the construction of recombinant industrial organisms

15

(iii) NUMBER OF SEQUENCES: 52

20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

(2) INFORMATION FOR SEQ ID NO: 1:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

40

(iii) HYPOTHETICAL: YES

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GTAGCTGCGG CCGCCTCCGT CTTCACTTCT TCGCCCGCAC T
41

50

(2) INFORMATION FOR SEQ ID NO: 2:

55

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

60

(iii) HYPOTHETICAL: YES

65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAAAGGGCAT GCGGCCGTAT CGGCCGGTGA CAAACATCAT TCAACGCC

48

(2) INFORMATION FOR SEQ ID NO: 3:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

20 ATGTTTAAGC TTGGCCGATA CGGCCAAAC ACCTTGATT TC
42

(2) INFORMATION FOR SEQ ID NO: 4:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

40 CAAGTTGCGG CCGCTCCTCA CTAACGAGCC AGCAGATATC GATGG
45

(2) INFORMATION FOR SEQ ID NO: 5:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

55 (iii) HYPOTHETICAL: YES

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

65 AAGCTTATGC GGCGCGAAT TCGAGCTCTG TACAGTGAC
39

(2) INFORMATION FOR SEQ ID NO: 6:

65 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

5 (iii) HYPOTHETICAL: YES

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGGTACGTGC GGCGCTCGT ACCATGGT GAGTGGTATG
40

15 (2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
20 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
25 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

35 ATATGTGCGG CCGCTTTACA TGGTCAATGC AATTAGATGG TGG
43

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

55 ATAACTCTAG AGGCCCTACC GGCCTTGCA AATATACTGT AAGAACCC
47

(2) INFORMATION FOR SEQ ID NO: 9:

60 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

65 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTATATTCTG CAGGGCCGGT AGGGCCAACA GTTTCCGCAG GTG
43

10 (2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

20 (iii) HYPOTHETICAL: YES

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTATGGGCGG CCGCTTTACA ACTAGAAATAT GGGAACCTGT GGG
43

30 (2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 61 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

40 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTCGAGTGCG GCCGCAAAGC TAGCTTGATA TCGAATTCCCT TATACTGGGC CTGCTGCATT
50 60

G
61

55 (2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

65 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

5 GTCCATATGG GTGTCTAGAA AAATAATGGT GAAAACTTGA AGGCG
45

(2) INFORMATION FOR SEQ ID NO: 13:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

25 CATATGGCGG ACACGCCCGT ACCGATCTTC
30

(2) INFORMATION FOR SEQ ID NO: 14:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

45 ATGCATTGGC TCGTCATGAA GAGCCTATCA TCCGGCCTGC GGCTCGTTCT TCGC
54

(2) INFORMATION FOR SEQ ID NO: 15:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

60 (iii) HYPOTHETICAL: YES

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CAGCTACCCC GCTTGAGCAG ACATC
25

(2) INFORMATION FOR SEQ ID NO: 16:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

20 GTCAGGGAAAG AACACGAGGG CGCAG
25

(2) INFORMATION FOR SEQ ID NO: 17:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

40 CCCTCTCTTC GTCGTTGTCC ACGCC
25

(2) INFORMATION FOR SEQ ID NO: 18:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

55 (iii) HYPOTHETICAL: YES

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

65 ATGTCCTTGG CCGACTTCAG CTCGG
25

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

5 (iii) HYPOTHETICAL: YES

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GACGAGCCAA TGCATCTTT GTATG
25

15 (2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
20 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
25 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CGGGTACTCG CTCTACCTAC TTCGG
35 25

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

55 GCCCAGTATA AGGAATTCGA TATCAAG
27

(2) INFORMATION FOR SEQ ID NO: 22:

60 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

65 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AGGGTCGACA CTAGTTCTAG AGCGG
25

10 (2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
15 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
20 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GACGTTATCG GACGGAGACT CAGTG
25

30 (2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
35 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
40 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GCCTACTCTG TTCTGGAGAG CTGC
50 24

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
55 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

60 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCCCCATCCC GGTACGGCAC TCGCG
25

5 (2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

15 (iii) HYPOTHETICAL: YES

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CACAGAGAAT GTGCCGTTTC TTTGG
25

25 (2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

35 (iii) HYPOTHETICAL: YES

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

TCACATATCC CCTACTCCCG AGCCG
25

45 (2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
50 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

55 (iii) HYPOTHETICAL: YES

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTCGCGTATC CCAGG
65 15

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "oligonucleotide"

10 (iii) HYPOTHETICAL: YES

10

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

18 GTCAAAGGAT ATGCATAC

20 (2) INFORMATION FOR SEQ ID NO: 30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "oligonucleotide"

30 (iii) HYPOTHETICAL: YES

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

39 AGCTTATGCG GCCGCGAATT CAGGTACCGT ATCTCGAGA

40 (2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "oligonucleotide"

50 (iii) HYPOTHETICAL: YES

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

39 AATTTCTCGA GATACGGTAC CTGAATTGCG GGCCGCATA

60 (2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

10 GTGCGAGGTA CCACAATCAA TCCATTTCGC
30

(2) INFORMATION FOR SEQ ID NO: 33:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

25 (iii) HYPOTHETICAL: YES

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

30 ATGGTTCAAG AACTCGGTAG CCTTTTCCTT GATTCT
36

35 (2) INFORMATION FOR SEQ ID NO: 34:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

45 (iii) HYPOTHETICAL: YES

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

50 AGAATCAAGG AAAAGGCTAC CGAGTTCTTG AACCAT
36

55 (2) INFORMATION FOR SEQ ID NO: 35:

55 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

60 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

65 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

5 ATCAATCAGA AGCTTTCTCT CGAGACGGGC ATCGGAGTCC CG
42

(2) INFORMATION FOR SEQ ID NO: 36:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

25 GACCATGATT ACGCCAAGCT T
21

(2) INFORMATION FOR SEQ ID NO: 37:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

45 GGATCCTTAA CTAGTTAAGT GGGGGCCTGC GCAAAG
36

(2) INFORMATION FOR SEQ ID NO: 38:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

60 (iii) HYPOTHETICAL: YES

65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

TTAACTAGTT AAGGATCCAC AATCAATCCA TTTCGC
36

(2) INFORMATION FOR SEQ ID NO: 39:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

20 GCTCTAGAGC GGCGCGAAT TCATCCGGAG ATCC
34

(2) INFORMATION FOR SEQ ID NO: 40:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

40 CTTTGCGCAG GCCCCCAC
18

(2) INFORMATION FOR SEQ ID NO: 41:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

55 (iii) HYPOTHETICAL: YES

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

TGCAGGGTAA ATCAGGGA
18

(2) INFORMATION FOR SEQ ID NO: 42:

65 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

5 (iii) HYPOTHETICAL: YES

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

TCCGCTAAAG GTGGTCGCG
19

15 (2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
20 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
25 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

CCCCAGCATC ATTACACCTC
35 20

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

55 AAAGGACCCG AGATCCGTAC
20

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
60 (A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

65 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

TCTCGATACC AAGGTCACCA CGGGC
25

10 (2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

20 (iii) HYPOTHETICAL: YES

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

GCATCCATCG GCCACCGTCA TTGGA
25

30 (2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: other nucleic acid

40 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

ATCCAGACCA GCACAGGCAG CTTCG
50 25

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

55 (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

60 (iii) HYPOTHETICAL: YES

65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

TCCGCATGCC AGAAAGAGTC ACCGG
25

5 (2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
10 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

15 (iii) HYPOTHETICAL: YES

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

GTCGACTTAA CTAGTTAAGG CTTCAGACGC AGCGAG
36

25 (2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
30 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

35 (iii) HYPOTHETICAL: YES

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

TTAACTAGTT AAGTCGACAC AATCAATCCA TTTCGC
36

45 (2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
50 (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

55 (iii) HYPOTHETICAL: YES

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

AGATCTTAA CTAGTTAAGT GGCTGAACA GTGCCG
65 36

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

- (ii) MOLECULE TYPE: other nucleic acid
 - (A) DESCRIPTION: /desc = "oligonucleotide"

- (iii) HYPOTHETICAL: YES

10

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

15

TTAACTAGTT AAAGATCTAC AATCAATCCA TTTCGC

36

CLAIMS

1. A filamentous fungus which has integrated a recombinant
5 DNA molecule into at least two substantially homologous
DNA domains of its chromosome(s), wherein the DNA
domains are not the ribosomal DNA repeats.
2. A filamentous fungus of claim 1, wherein a recombinant
10 DNA molecule is integrated into each of its
substantially homologous DNA domains.
3. The filamentous fungus of claims 1 or 2, wherein said
DNA domains are amplicons.
15
4. The filamentous fungus of any one of claims 1 to 3,
wherein said DNA domain is a domain which in its native
state comprises an endogenous gene capable of high
level expression.
20
5. The filamentous fungus of claim 4, wherein said
endogenous gene is selected from the group consisting
of genes encoding glycolytic enzymes, amylolytic
enzymes, cellulolytic enzymes and antibiotic
25 biosynthetic enzymes.
6. The filamentous fungus of claim 5, wherein said
endogenous gene is selected from the group consisting
of a glucoamylase gene, a TAKA amylase gene, a
30 cellobiohydrolase gene and penicillium biosynthetic
genes.
7. The filamentous fungus of any one of claims 4 to 6,
wherein said endogenous gene is inactivated in each
35 copy of said DNA domain.
8. The filamentous fungus of claim 7, wherein said
endogenous gene is inactivated by means of an

irreversible deletion of at least part thereof.

9. The filamentous fungus of claim 8, wherein said irreversible deletion comprises at least part of the 5 promoter and upstream activating sequences.
10. A filamentous fungus of any one of claims 1 to 9, wherein each version of said DNA domains is distinguished from the other versions of the domains by 10 means of unique sequence tag.
11. The filamentous fungus of claim 10, wherein said sequence tag is a restriction site.
- 15 12. The filamentous fungus of any one of claims 1 to 11, wherein said recombinant DNA molecule contains an expression cassette for the expression of a desired gene.
- 20 13. The filamentous fungus of claim 12, wherein said desired gene encodes a secreted enzyme.
14. The filamentous fungus of claim 12, wherein said desired gene encodes an intracellular enzyme.
- 25 15. The filamentous fungus of claim 14, wherein one or more recombinant DNA molecules comprising one or more expression cassettes for intracellular enzymes are integrated into said DNA domain and wherein the 30 intracellular enzymes are part of a metabolic pathway which is not native to the filamentous fungus.
16. The filamentous fungus of claim 15, wherein the intracellular enzymes are selected from the group comprising a deacetoxycephalosporin C synthetase (expandase) and a deacetylcephalosporin C synthetase (hydroxylase).

17. The filamentous fungus of any one of claims 1 to 16, wherein the recombinant DNA molecule lacks a selectable marker gene.
- 5 18. The filamentous fungus of claim 17, which lacks a selectable marker gene.
- 10 19. The filamentous fungus of any one of claims 1 to 18, which belongs to a genus selected from the group consisting of *Aspergillus*, *Trichoderma*, *Penicillium*, *Cephalosporium*, *Acremonium*, *Fusarium*, *Mucor*, *Rhizophorus*, *Phanerochaete*, *Neurospora*, *Humicola*, *Claviceps*, *Sordaria*, *Ustilago*, *Schizophyllum*, *Blakeslea*, *Mortierella*, *Phycomyces*, and *Tolypocladium*.
- 15 20. The filamentous fungus of claim 19, which is selected from the group consisting of members of the *Aspergillus niger* group, *Aspergillus oryzae*, *Trichoderma reesei* and *Penicillium chrysogenum*.
- 20 21. A method for preparing a filamentous fungus as defined in any one of claims 1 to 20, wherein said method comprises the steps of:
 - (a) transforming a filamentous fungus comprising in one or more of its chromosomes at least two substantially homologous DNA domains suitable for integration of one or more copies of a recombinant DNA molecule and wherein the DNA domains are not the ribosomal DNA repeats, with a recombinant DNA molecule;
 - (b) selecting a transformant with at least one recombinant DNA molecule integrated in at least one of the DNA domains;
 - (c) propagating the transformant obtained in (b) and selecting from its progeny a strain in which at least two of the DNA domains comprise the integrated recombinant DNA molecule.

22. The method of claim 21, comprising in addition to steps (a), (b), and (c), the steps of:

5 (d) propagating the strain in which at least two of the DNA domains comprise an integrated DNA molecule and selecting from its progeny a strain in which additional copies of the DNA domains comprise the integrated recombinant DNA molecule;

10 (e) repeating step (d) until a strain is obtained in which each of the DNA domains comprises the integrated recombinant DNA molecule.

23. The method of claims 21 or 22, wherein the recombinant DNA molecule comprises sequences which are substantially homologous to the DNA domains.

15 24. The method of any one of claims 21 to 23, wherein a bidirectional selectable marker is used for transforming the filamentous fungus in step (a), and wherein, prior to step (c), transformants are selected for the absence of the bidirectional marker.

20 25. The method of claim 24, wherein the bidirectional marker is a dominant marker.

25 26. A method for production of a protein of interest, comprising the steps of:

30 (a) culturing a filamentous fungus according to any one of claims 1 to 20 under conditions conducive to the expression of the protein of interest; and (b) recovery of the protein of interest.

27. The method of claim 26, wherein the protein of interest is a secreted protein.

35 28. A method for the production of a metabolite of interest comprising the steps of:

(a) culturing a filamentous fungus of any one of claims 1 to 20 under conditions conducive to the

production of the metabolite of interest; and

(b) recovery of the metabolite of interest.

29. The method of claim 28, wherein the metabolite of
5 interest is a secondary metabolite.

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Visualisation of the Δ glaA loci of *A. niger* ISO-505

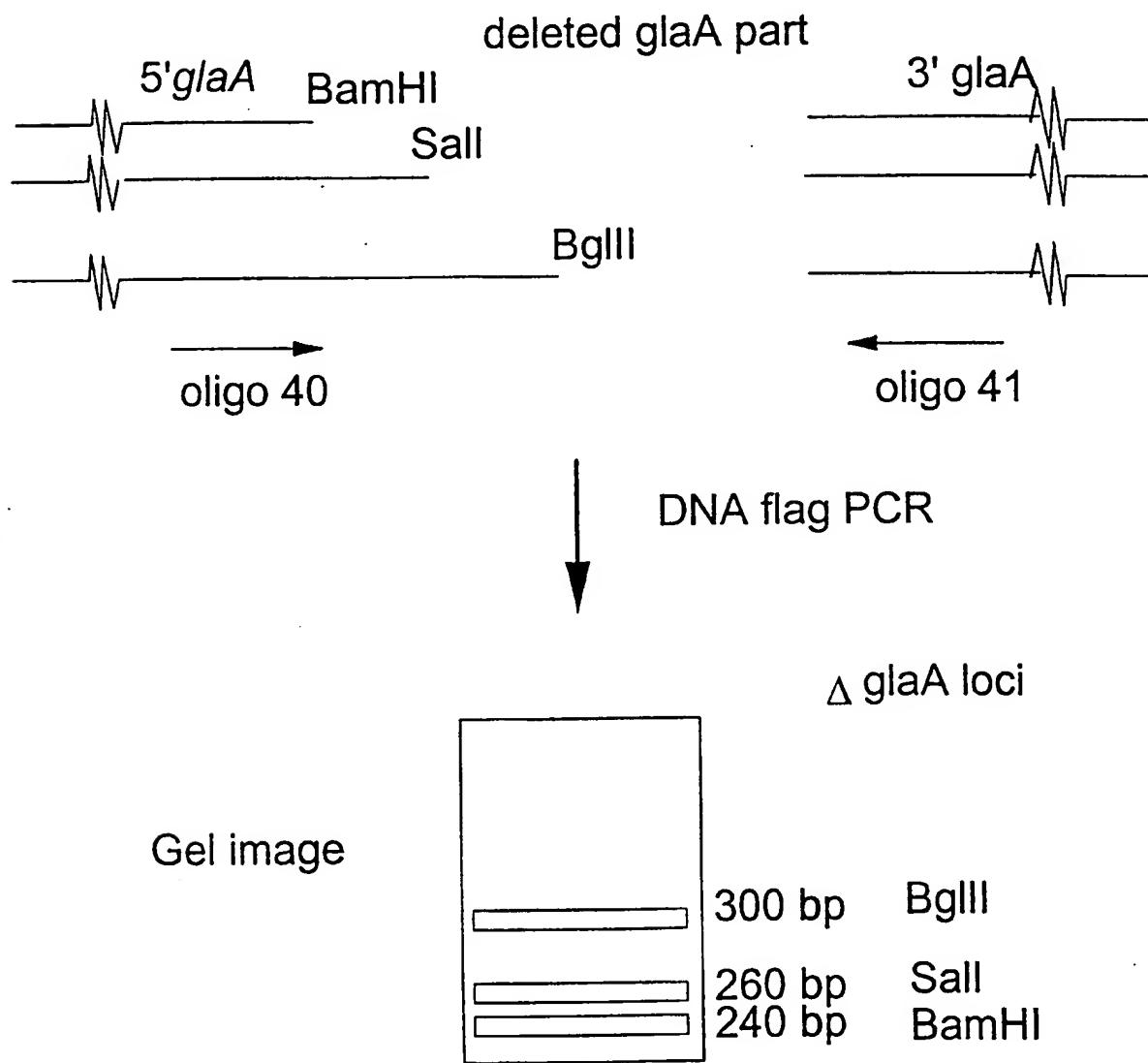


Figure 1

3

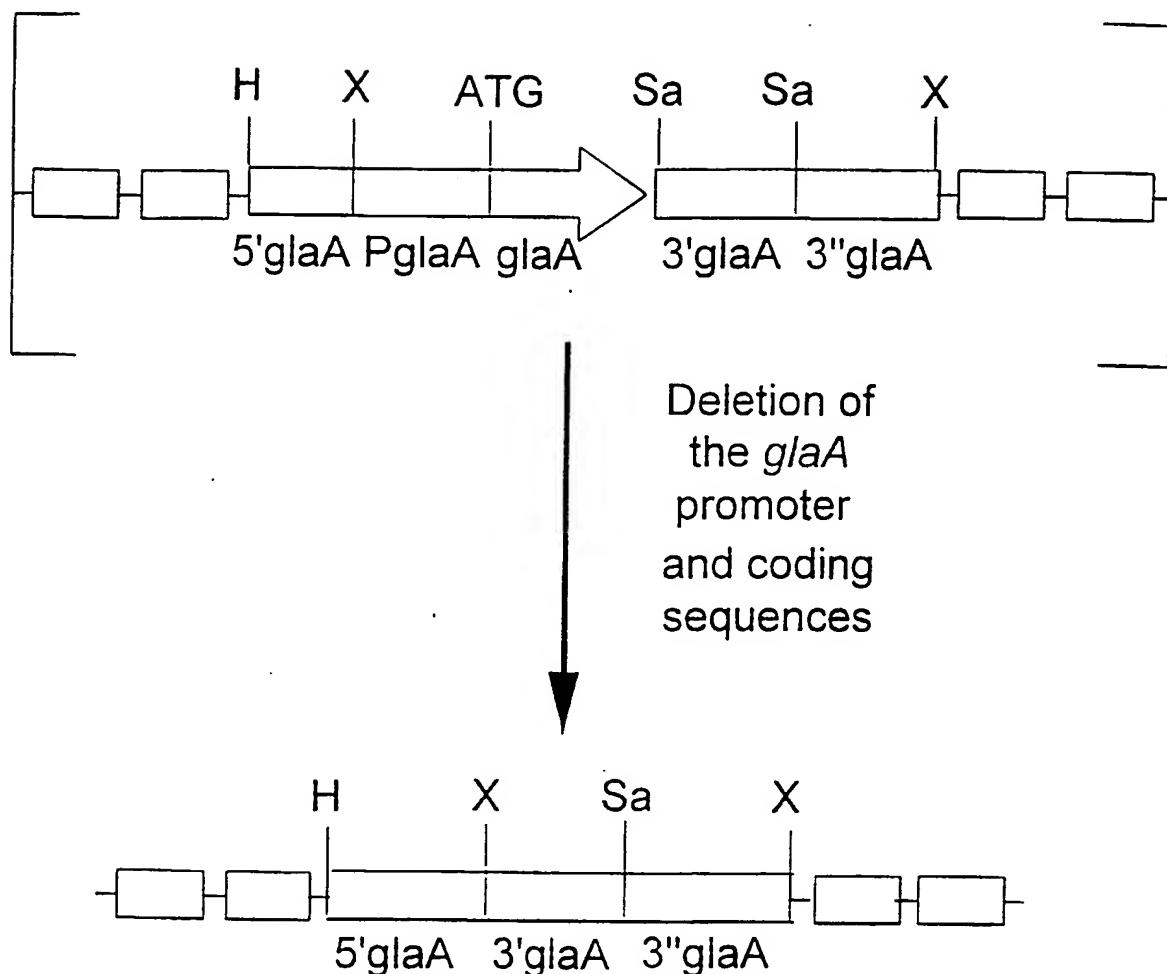


Figure 2

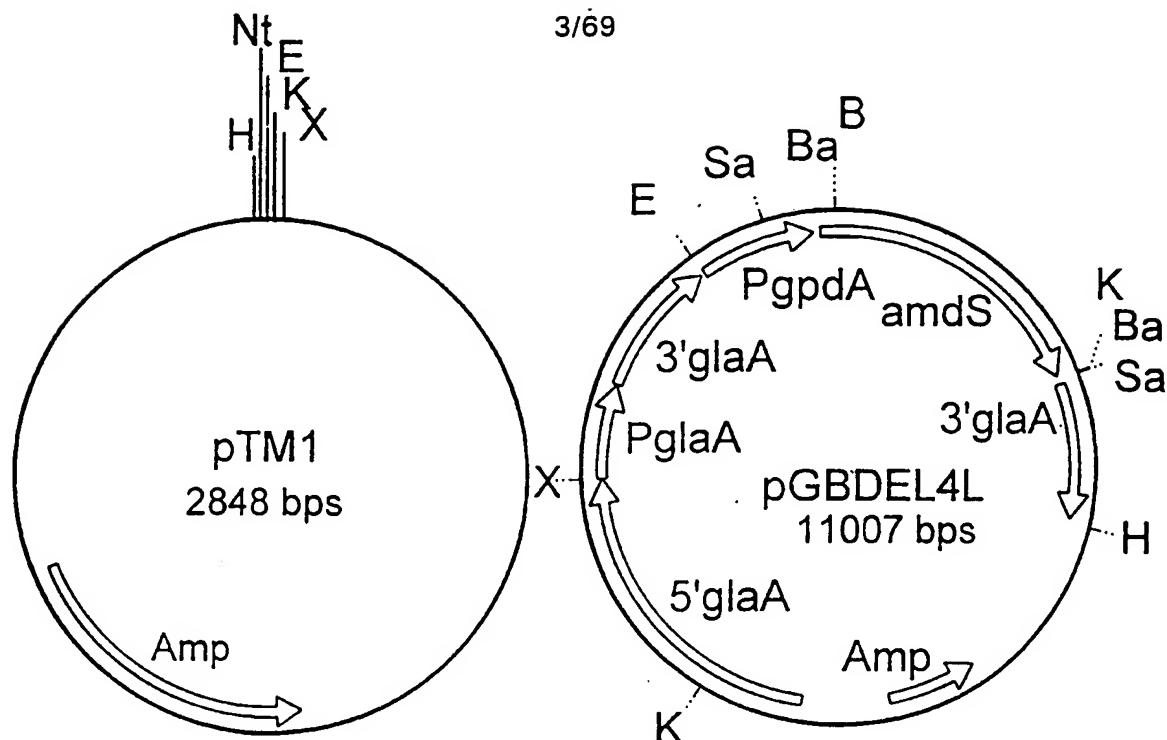


Figure 3a

EcoRI/KpnI
digestion

Ligation

EcoRI/KpnI/Xhol
digestion

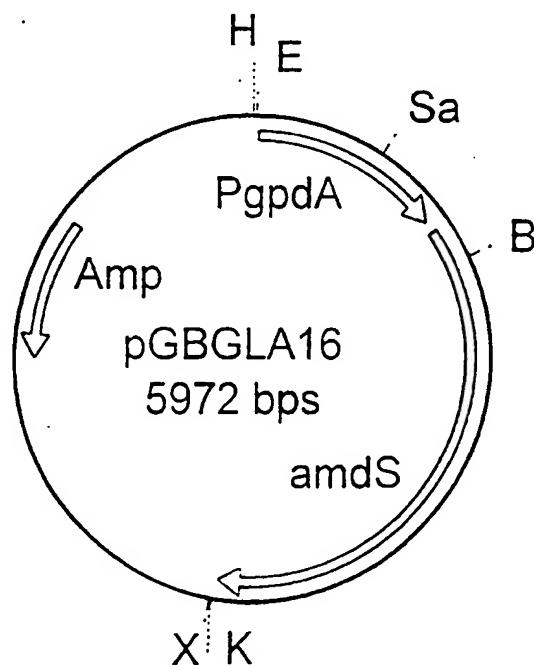


Figure 3b

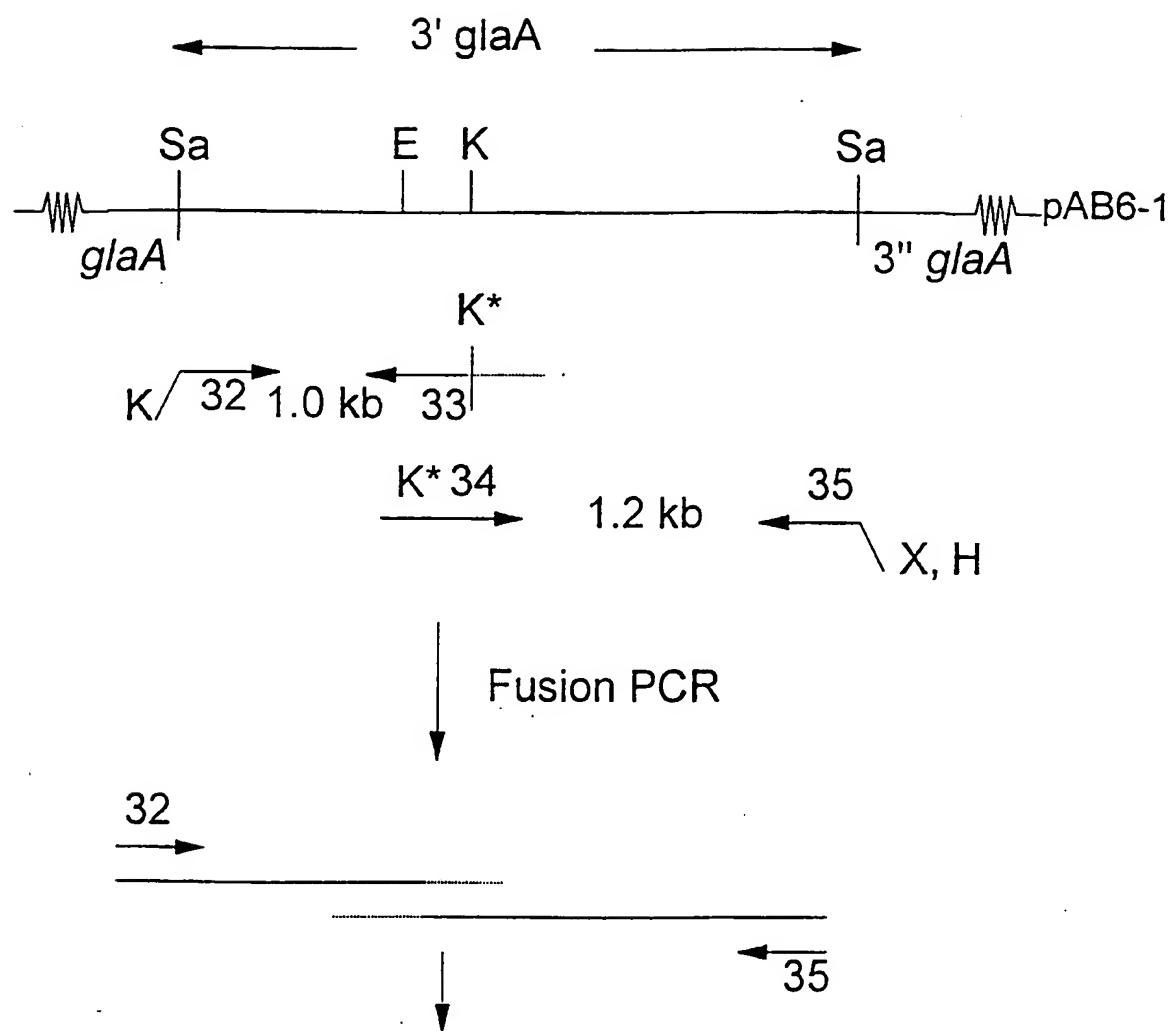


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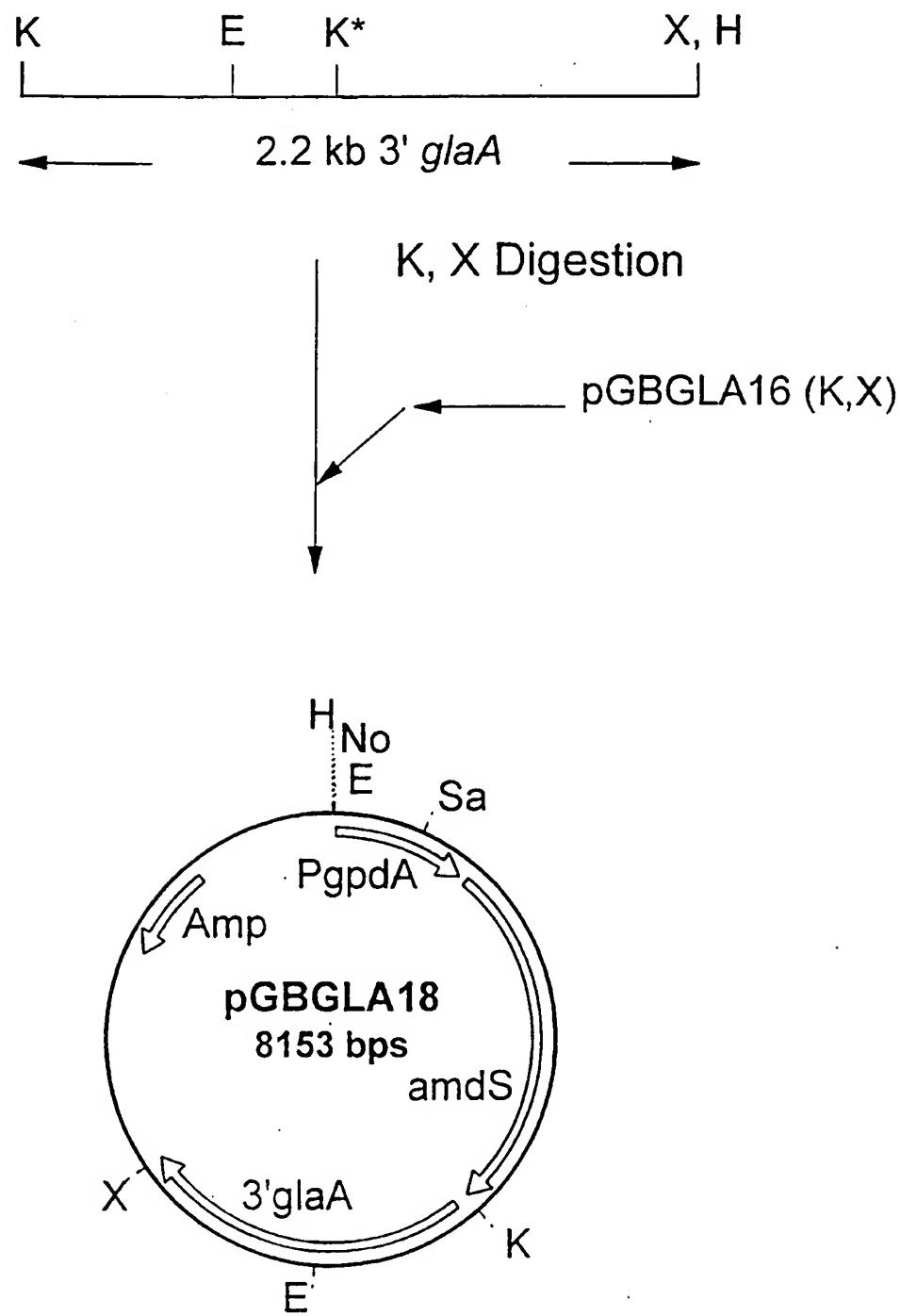


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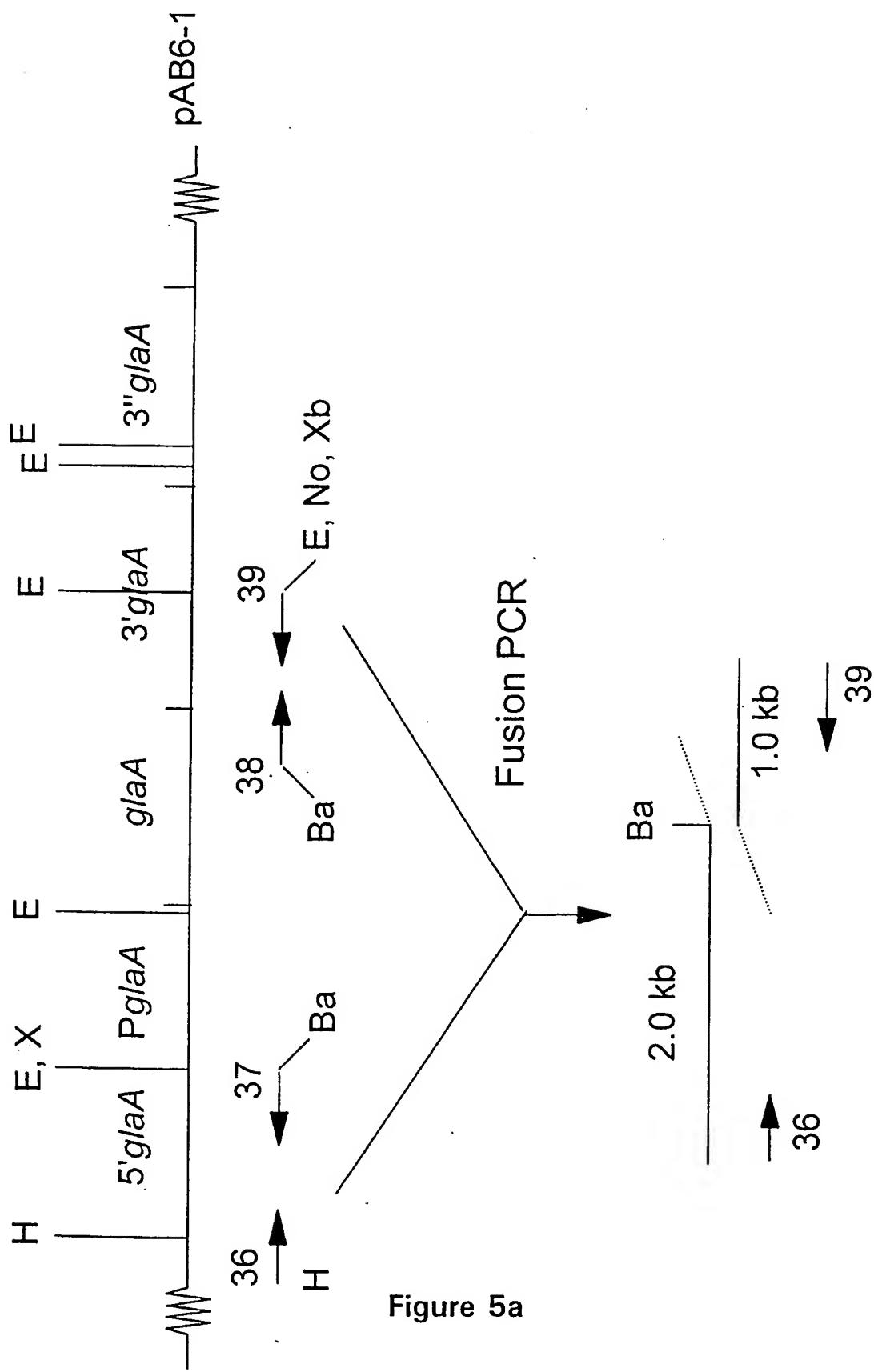


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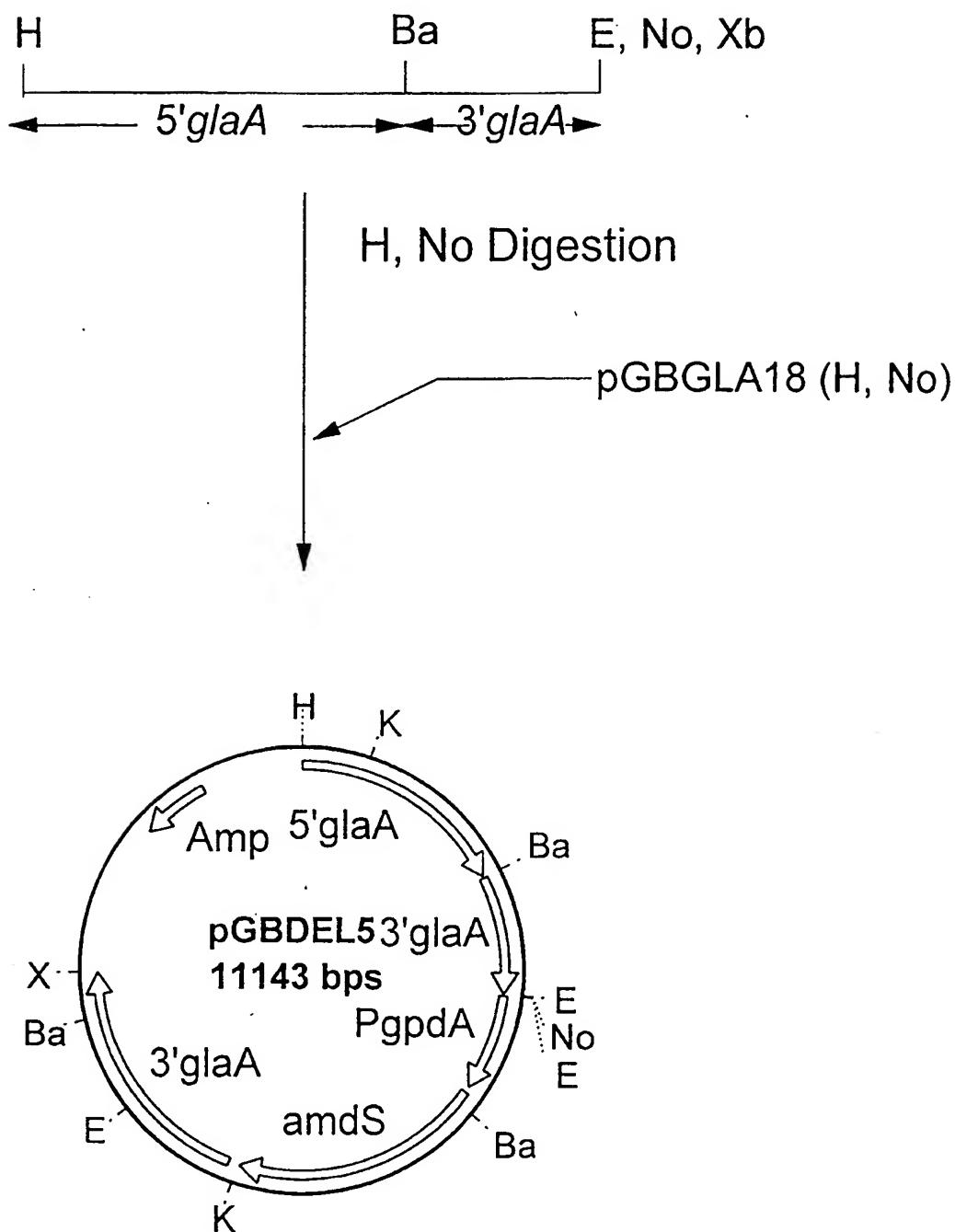


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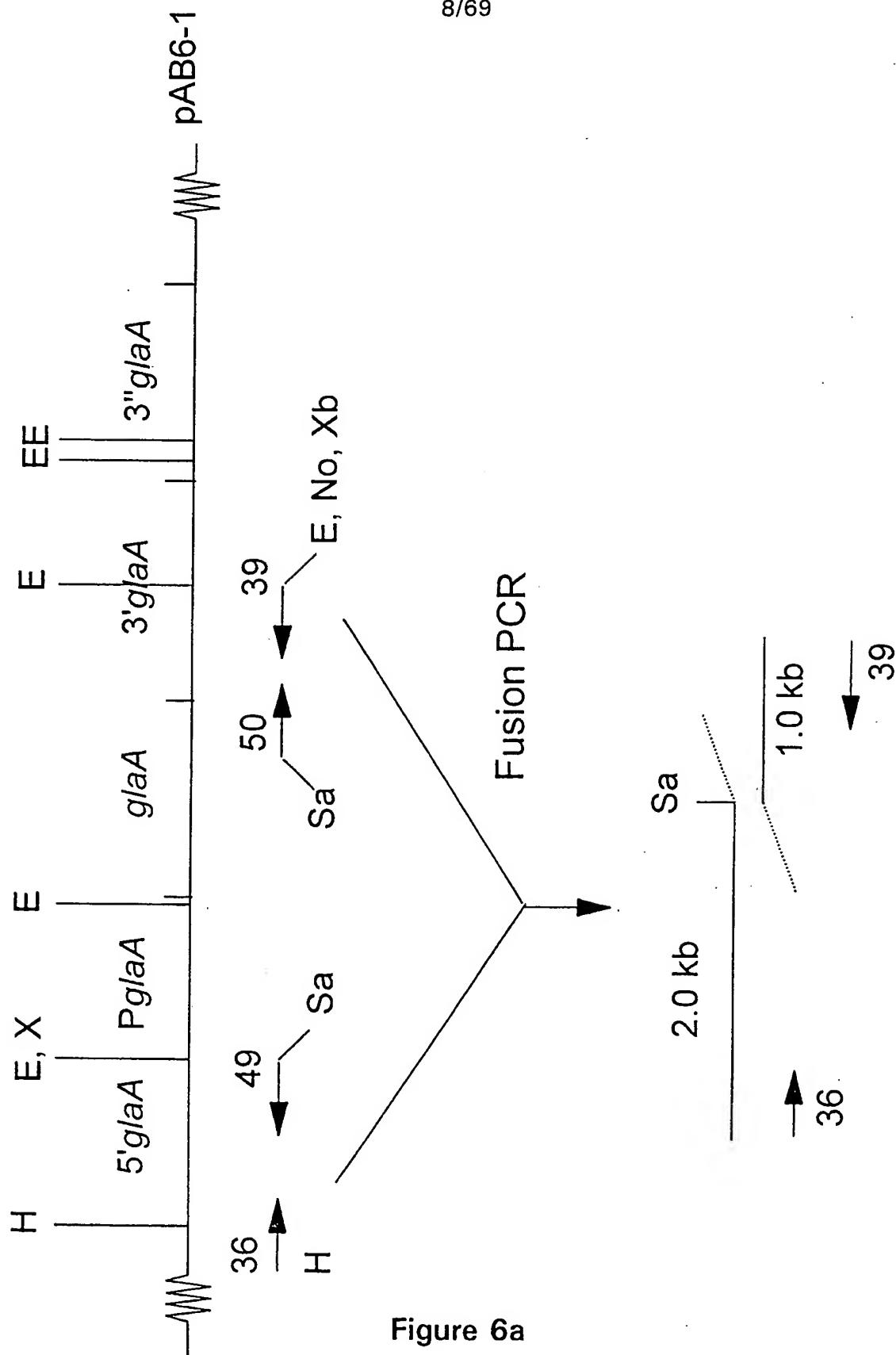


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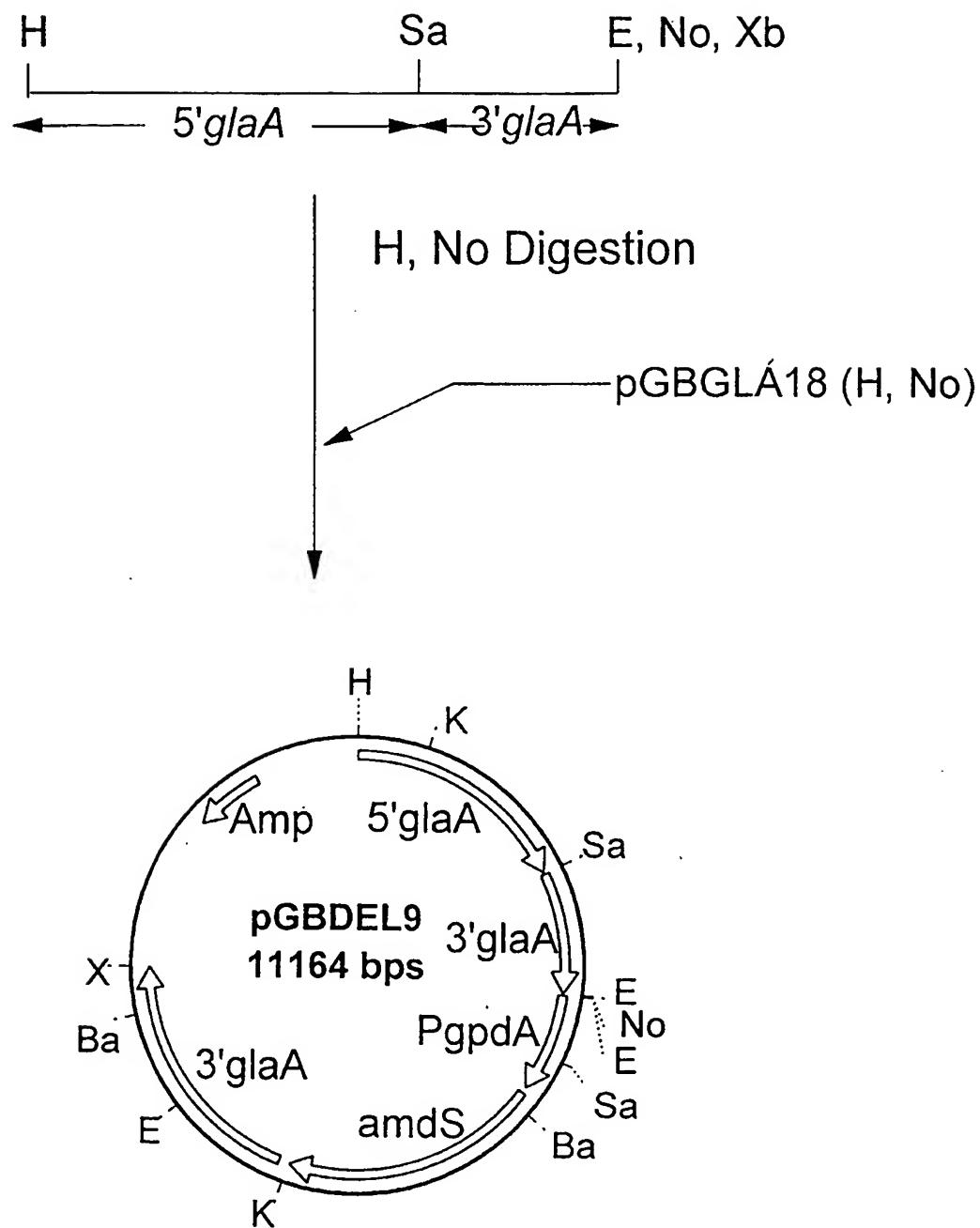


Figure 6b

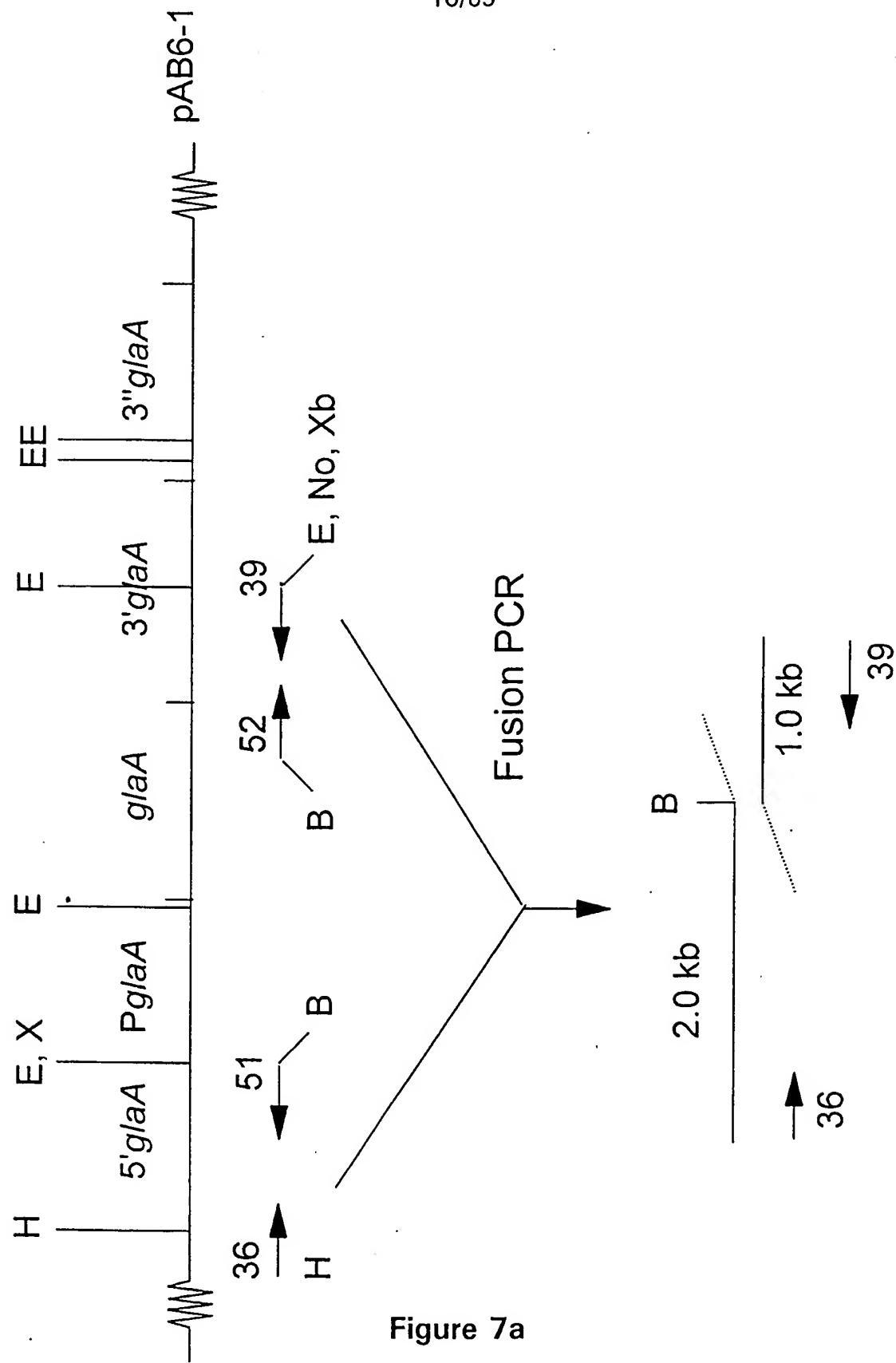


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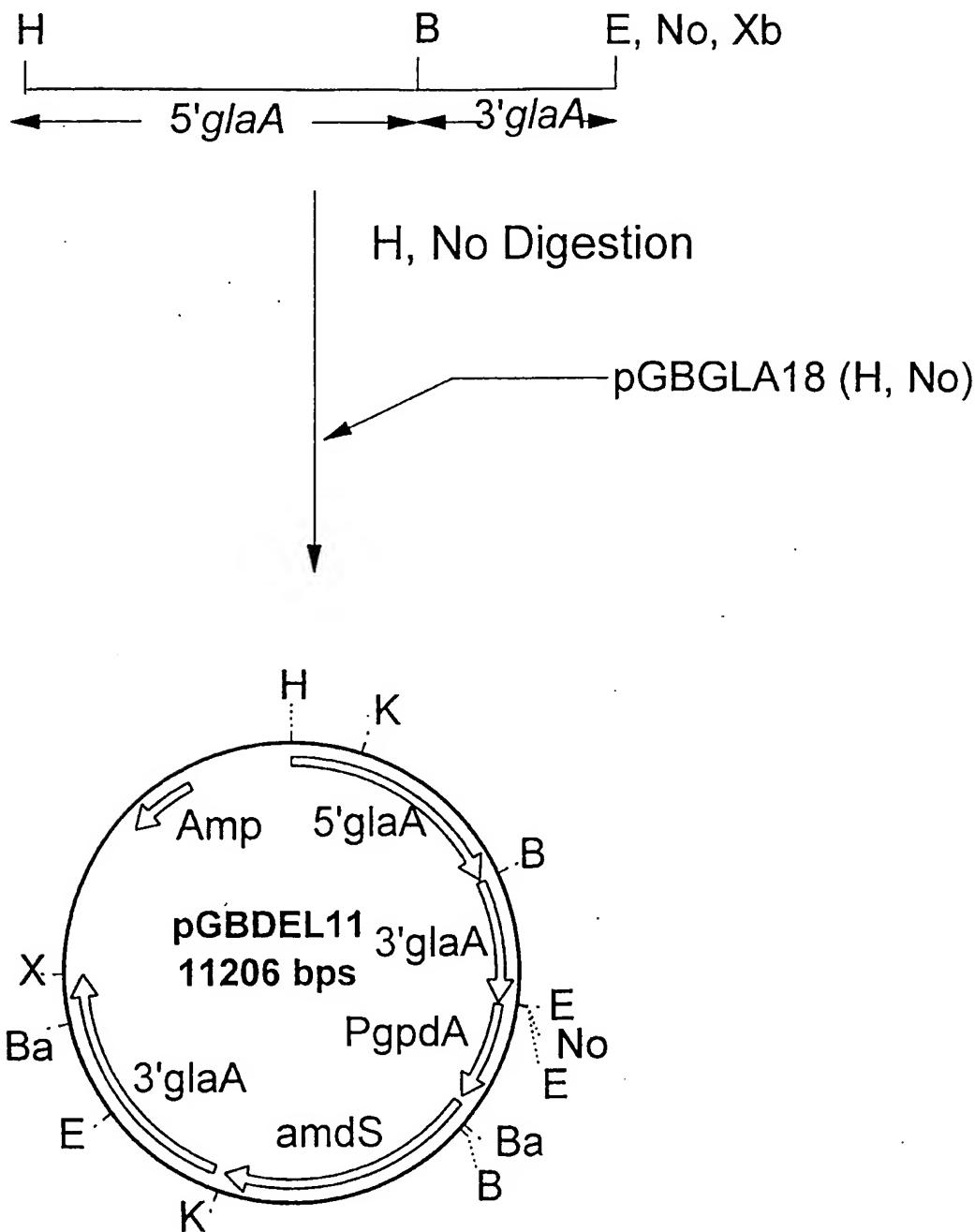


Figure 7b

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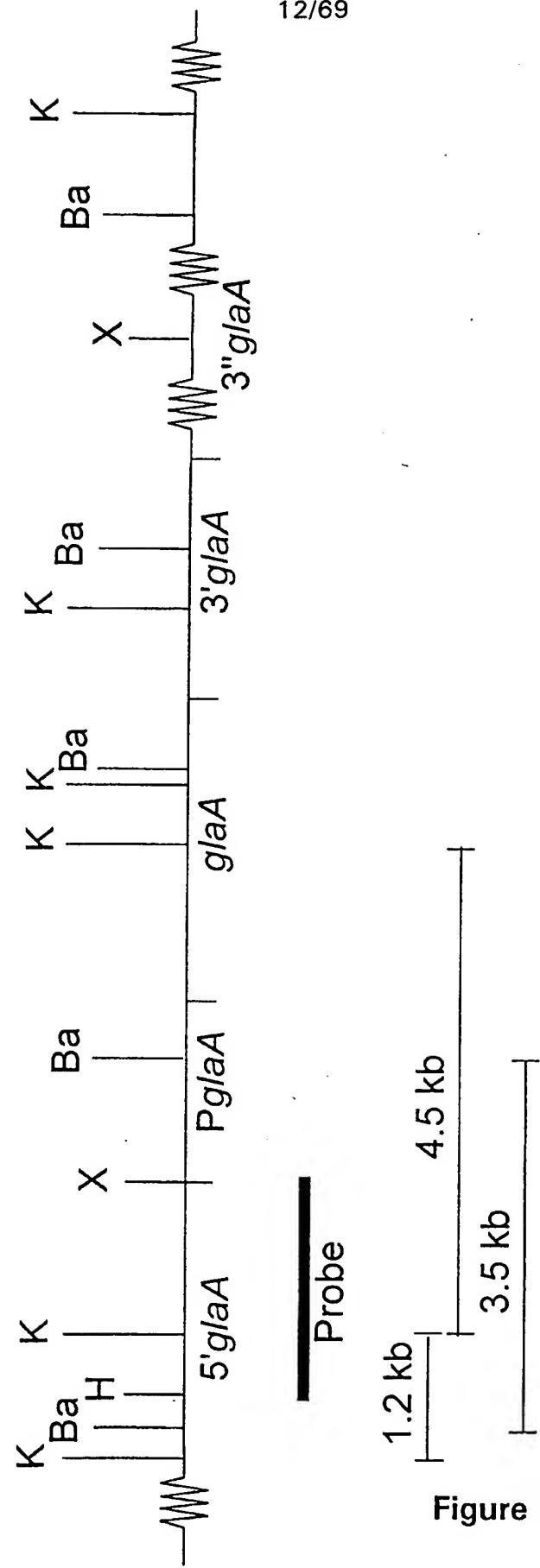


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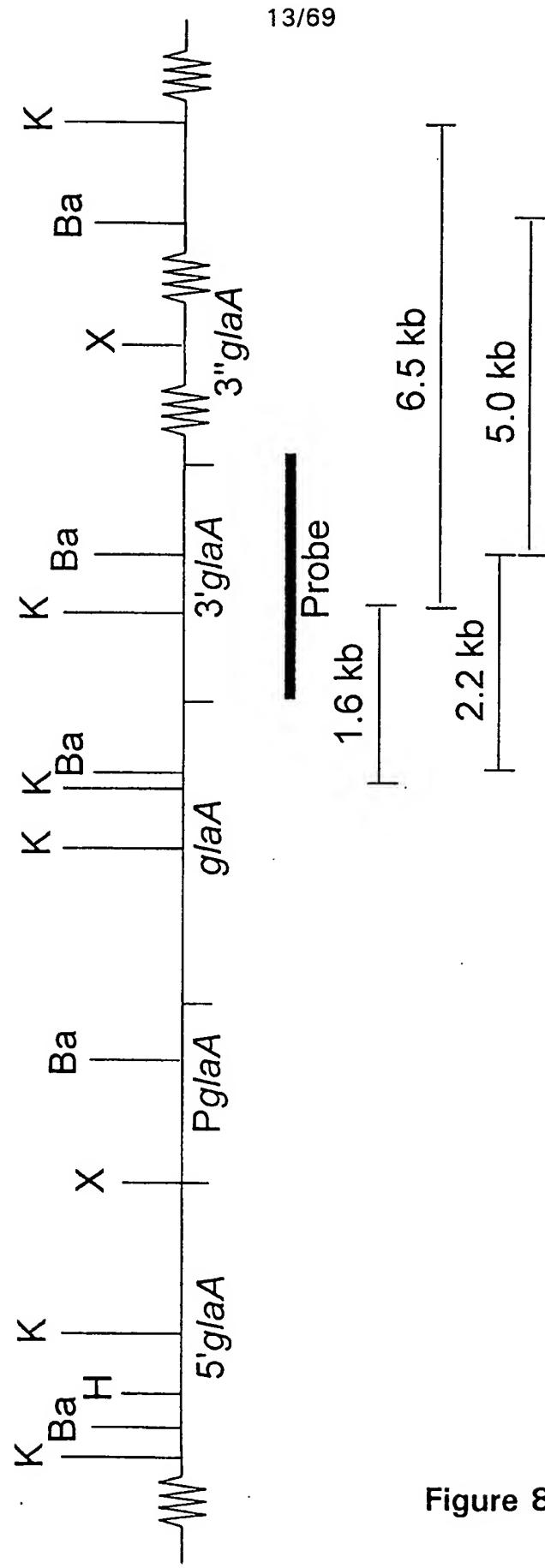


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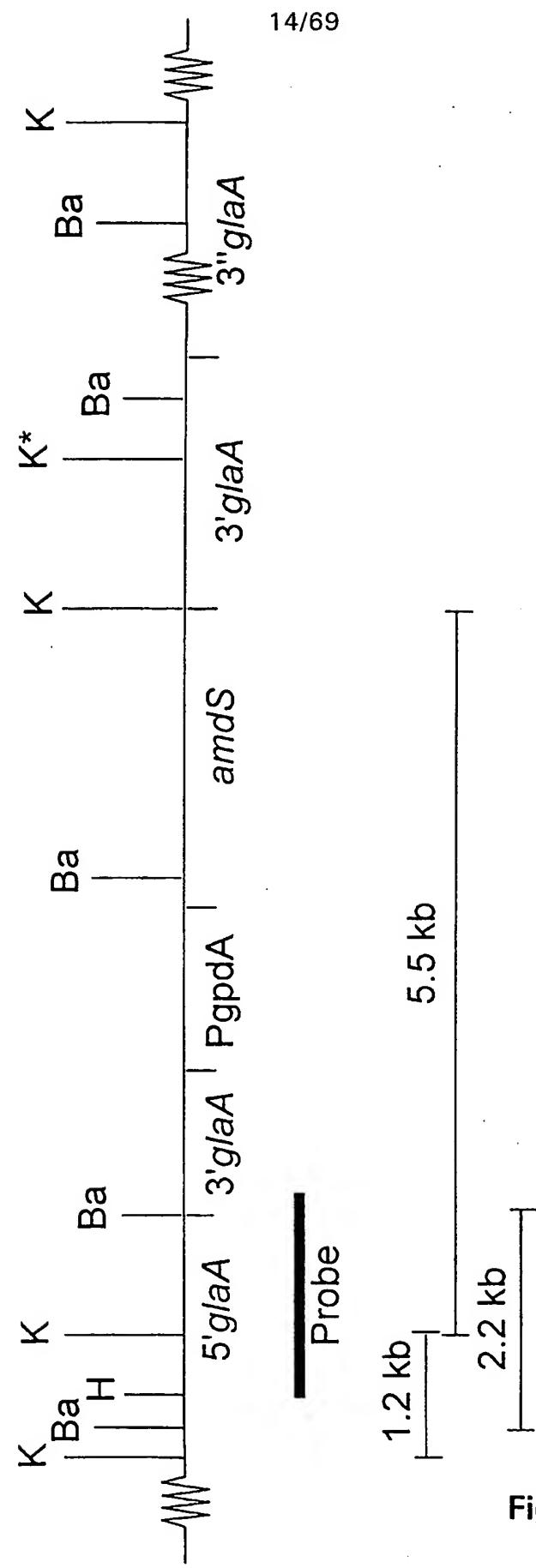


Figure 9a

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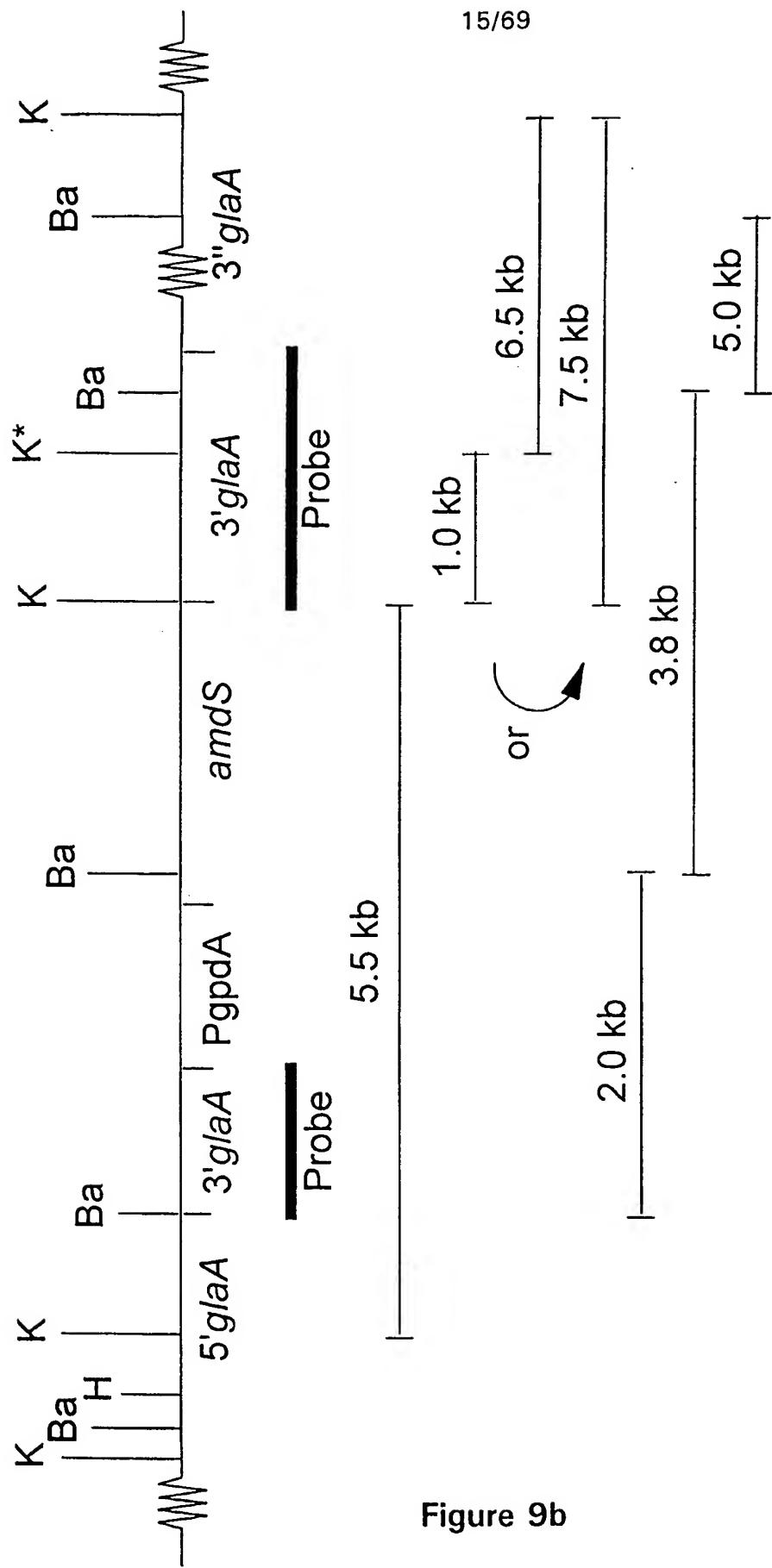


Figure 9b

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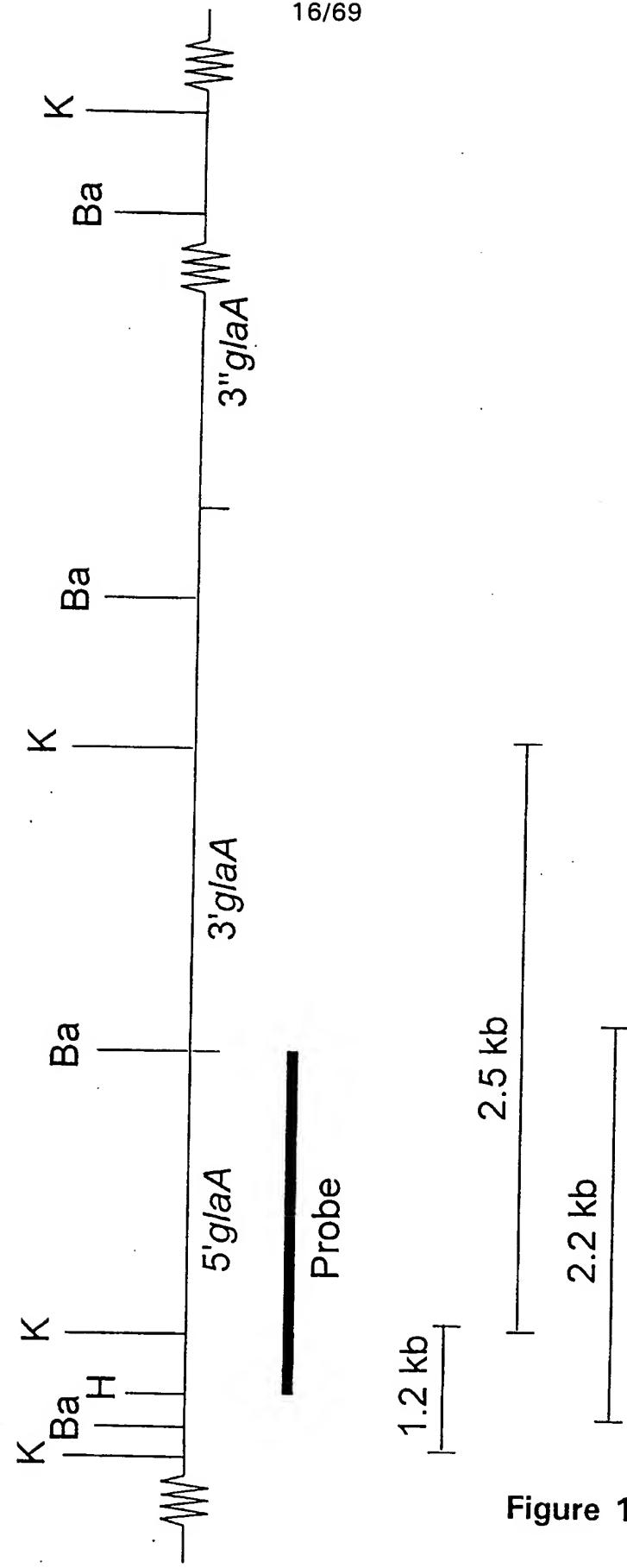


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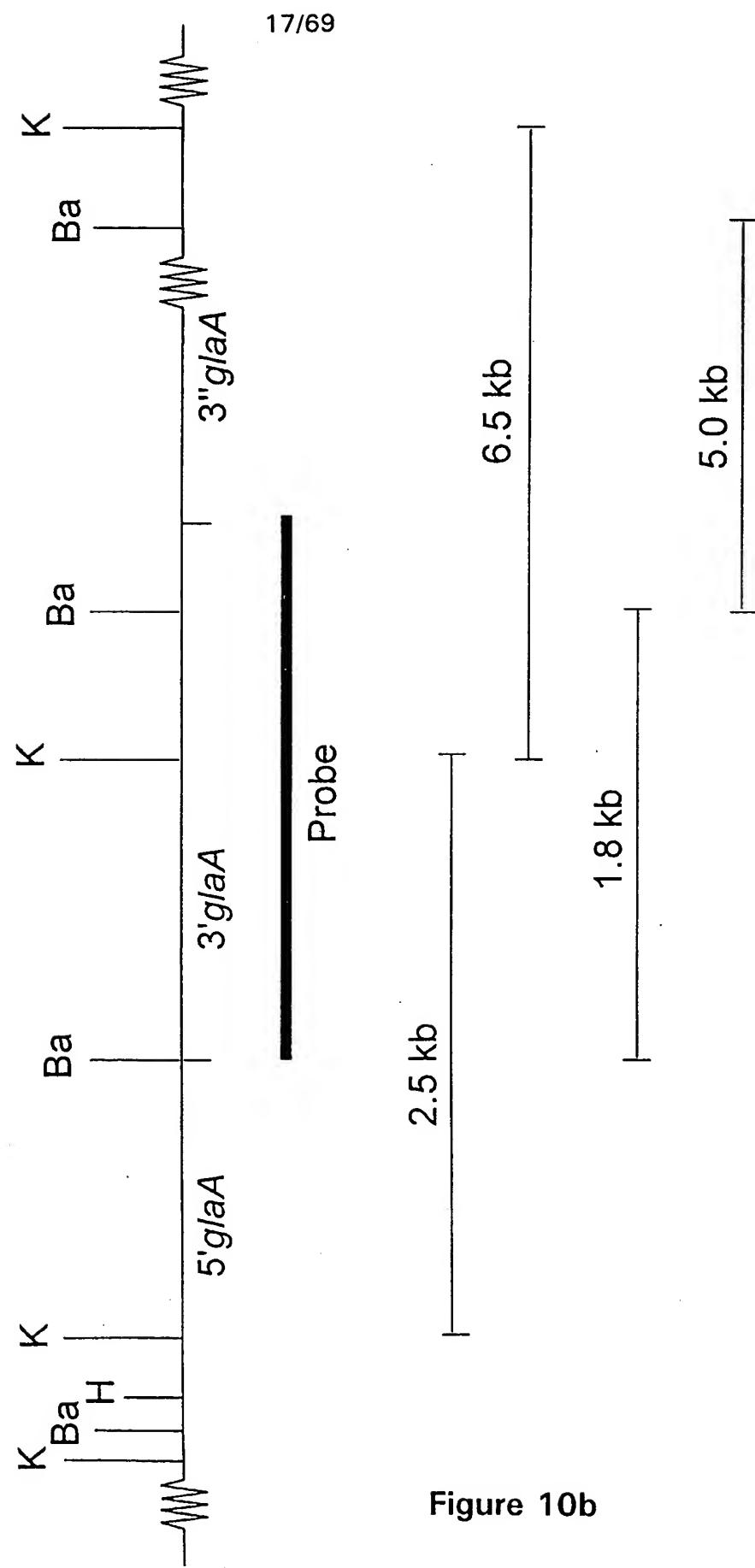


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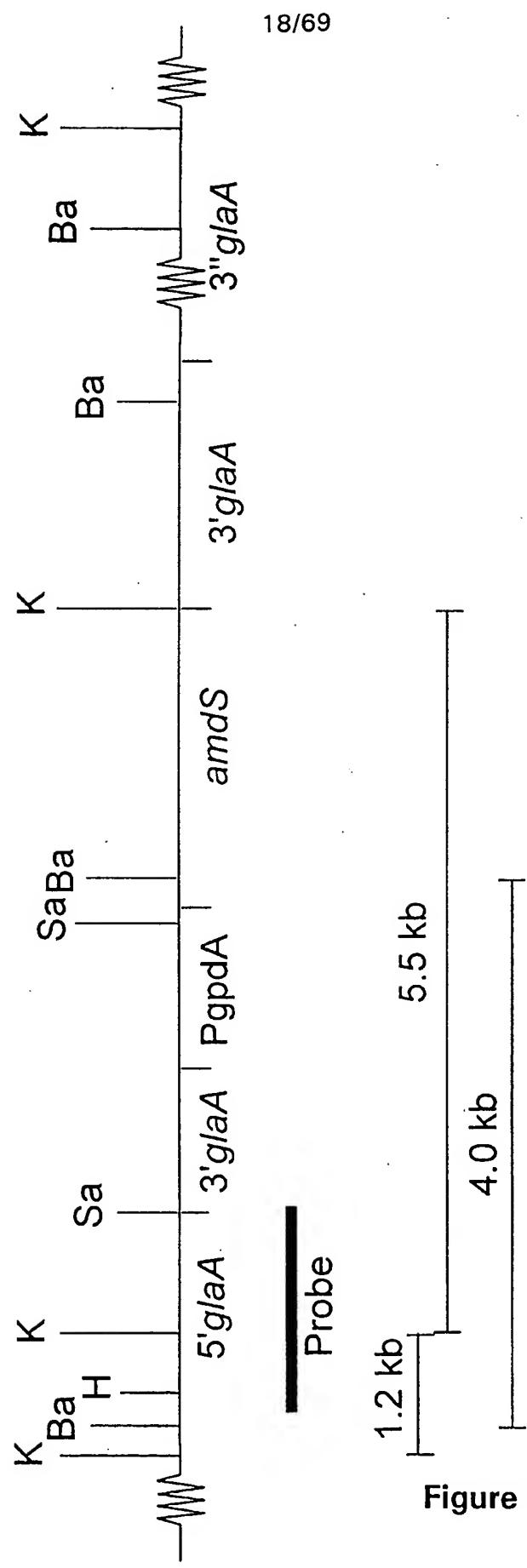


Figure 11a

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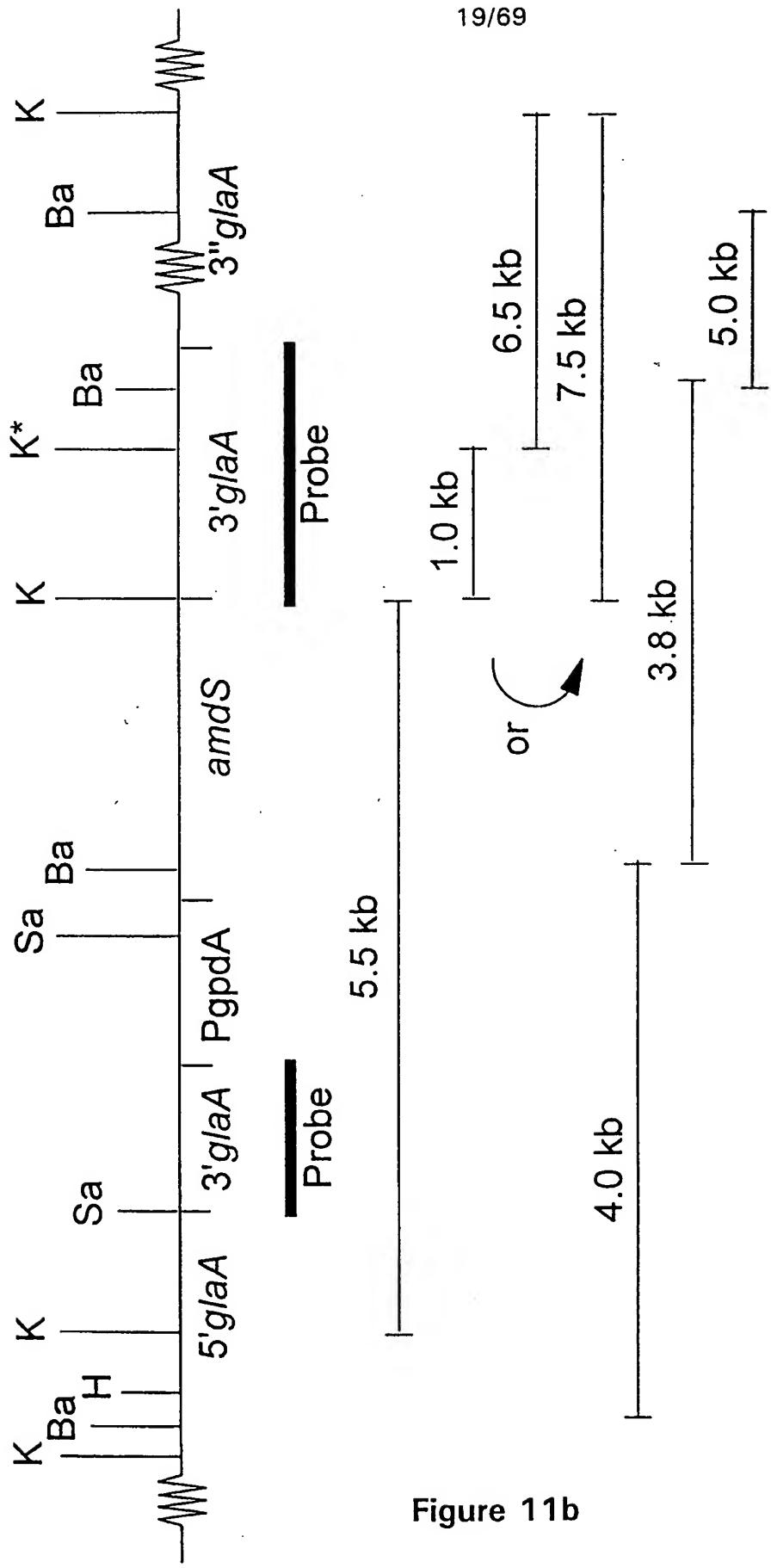


Figure 11b

20/69

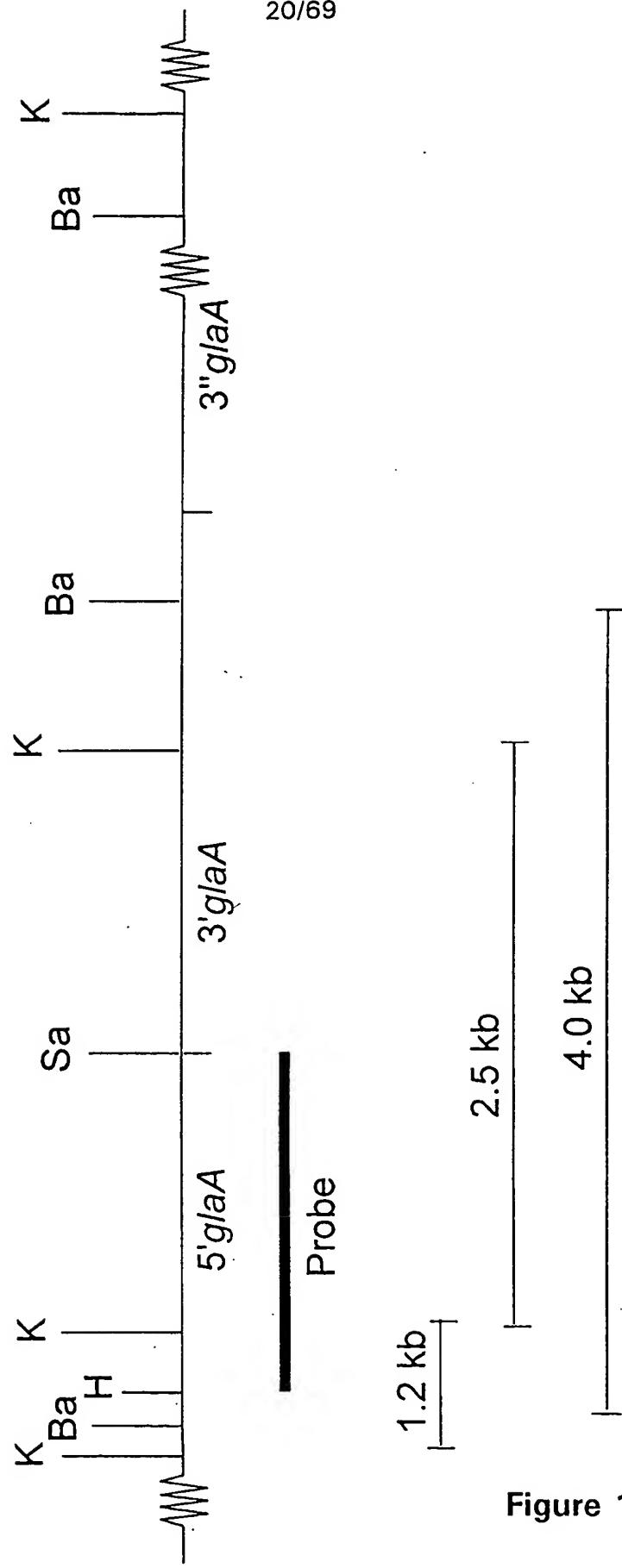


Figure 12a

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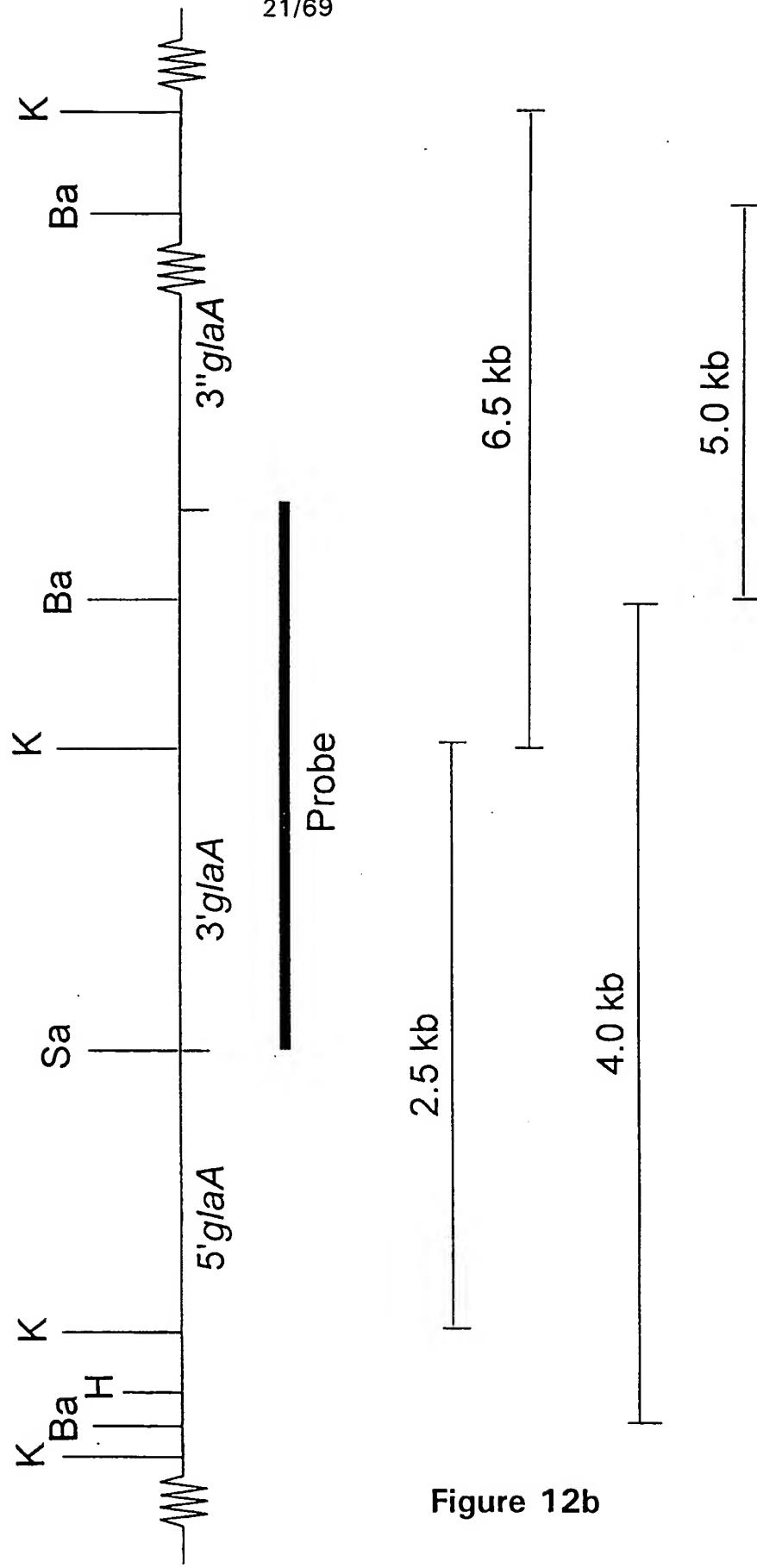


Figure 12b

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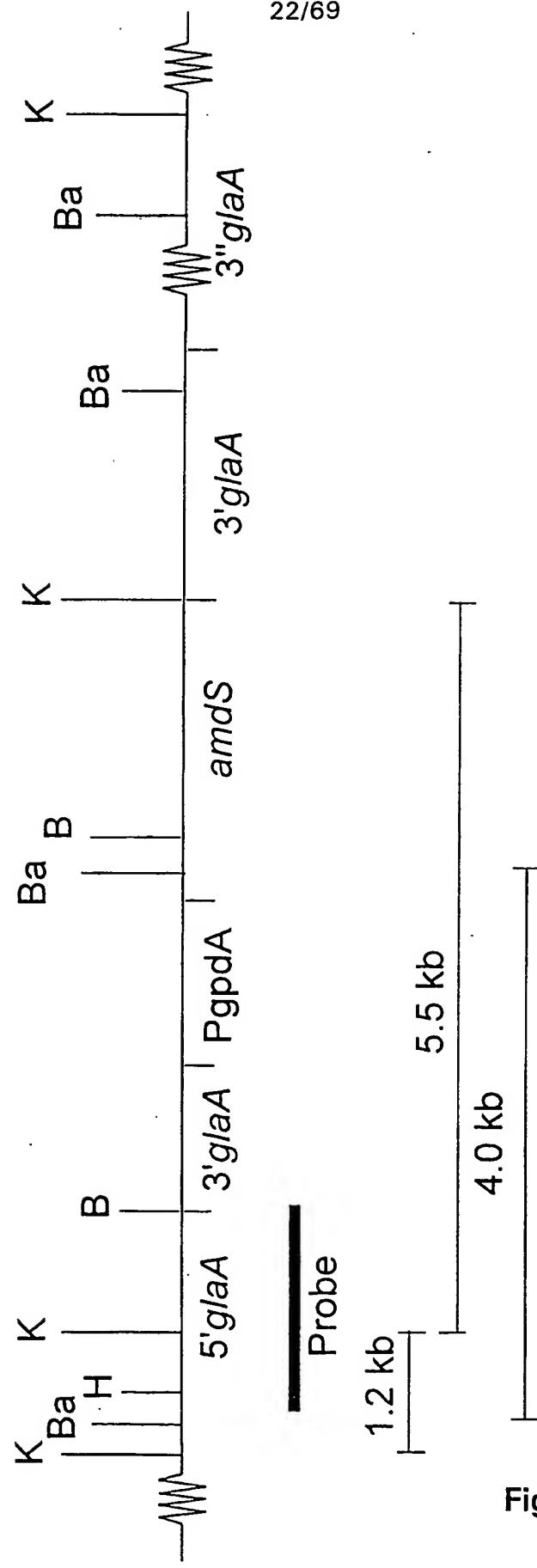


Figure 13a

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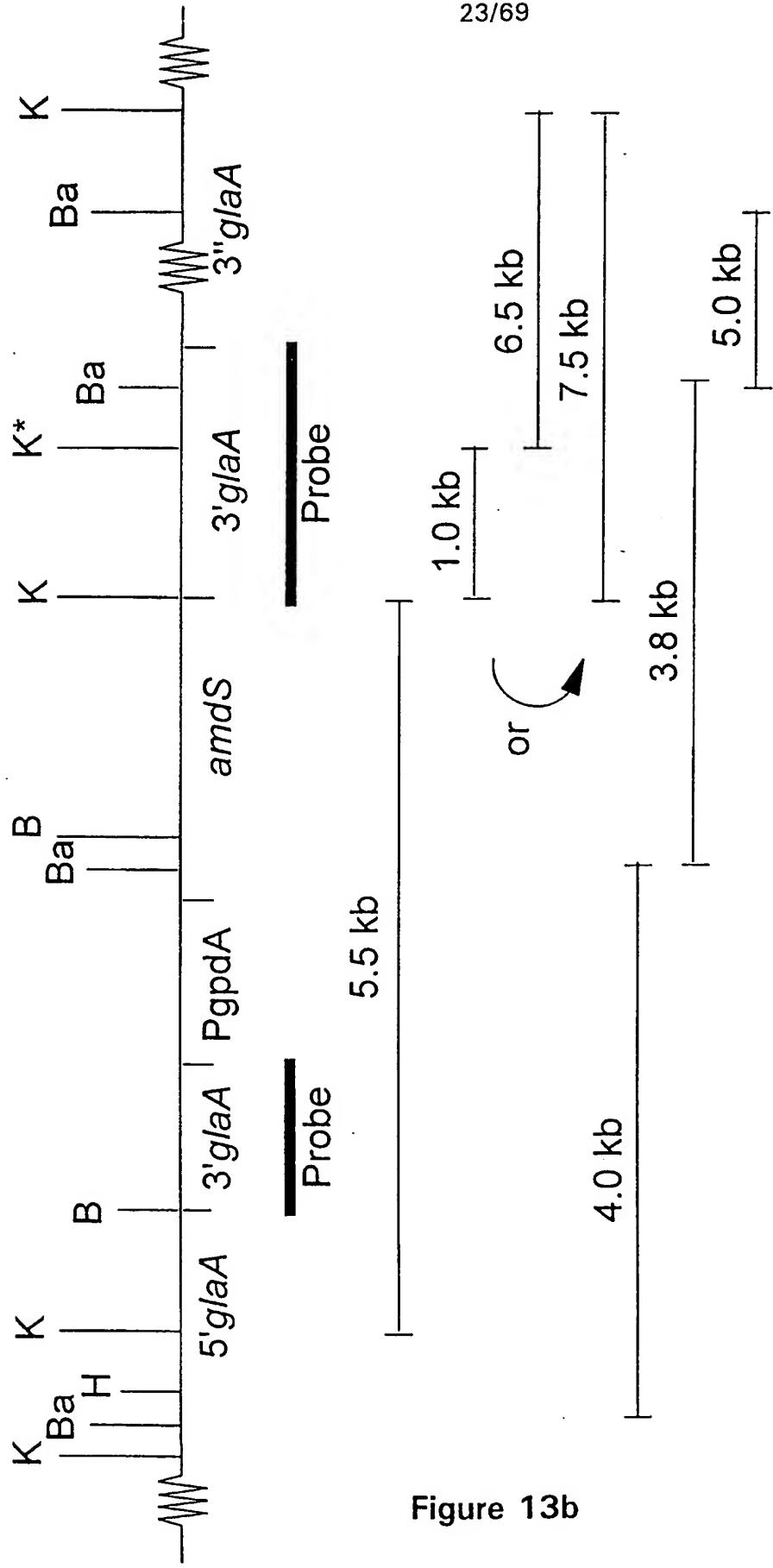


Figure 13b

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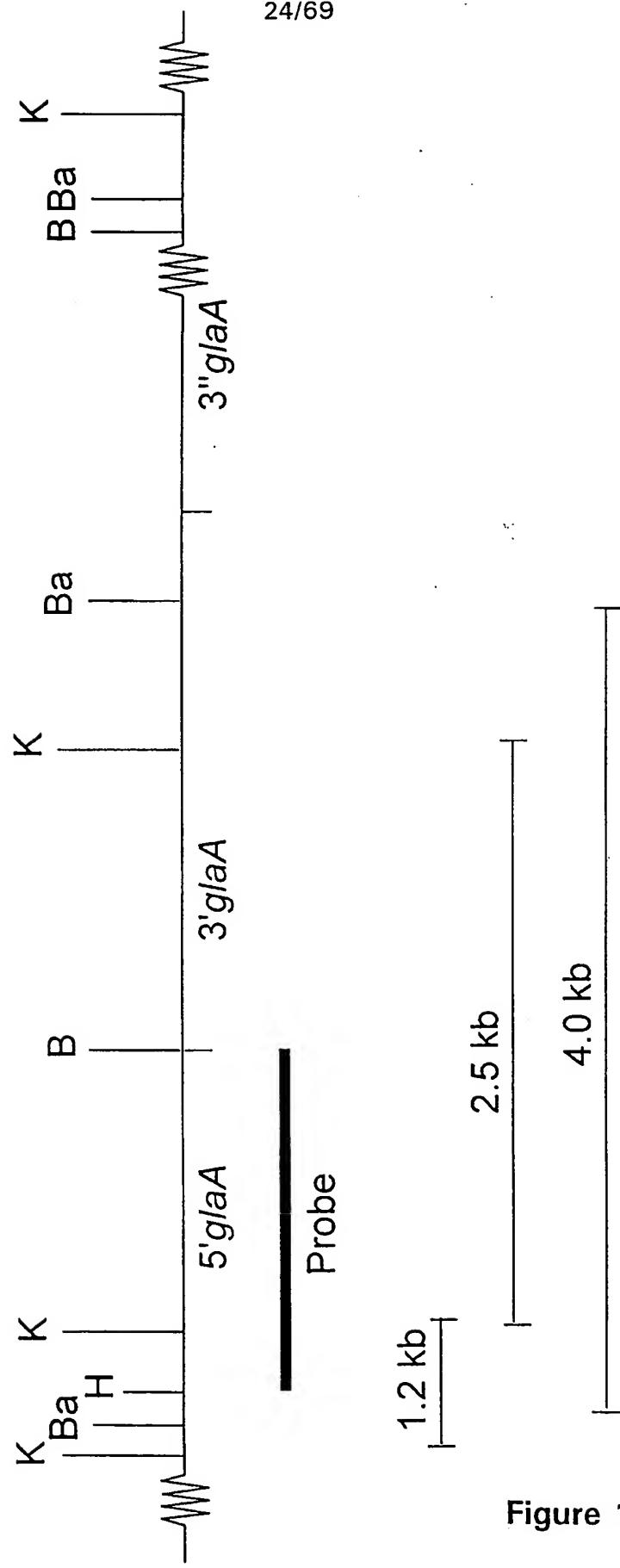


Figure 14a

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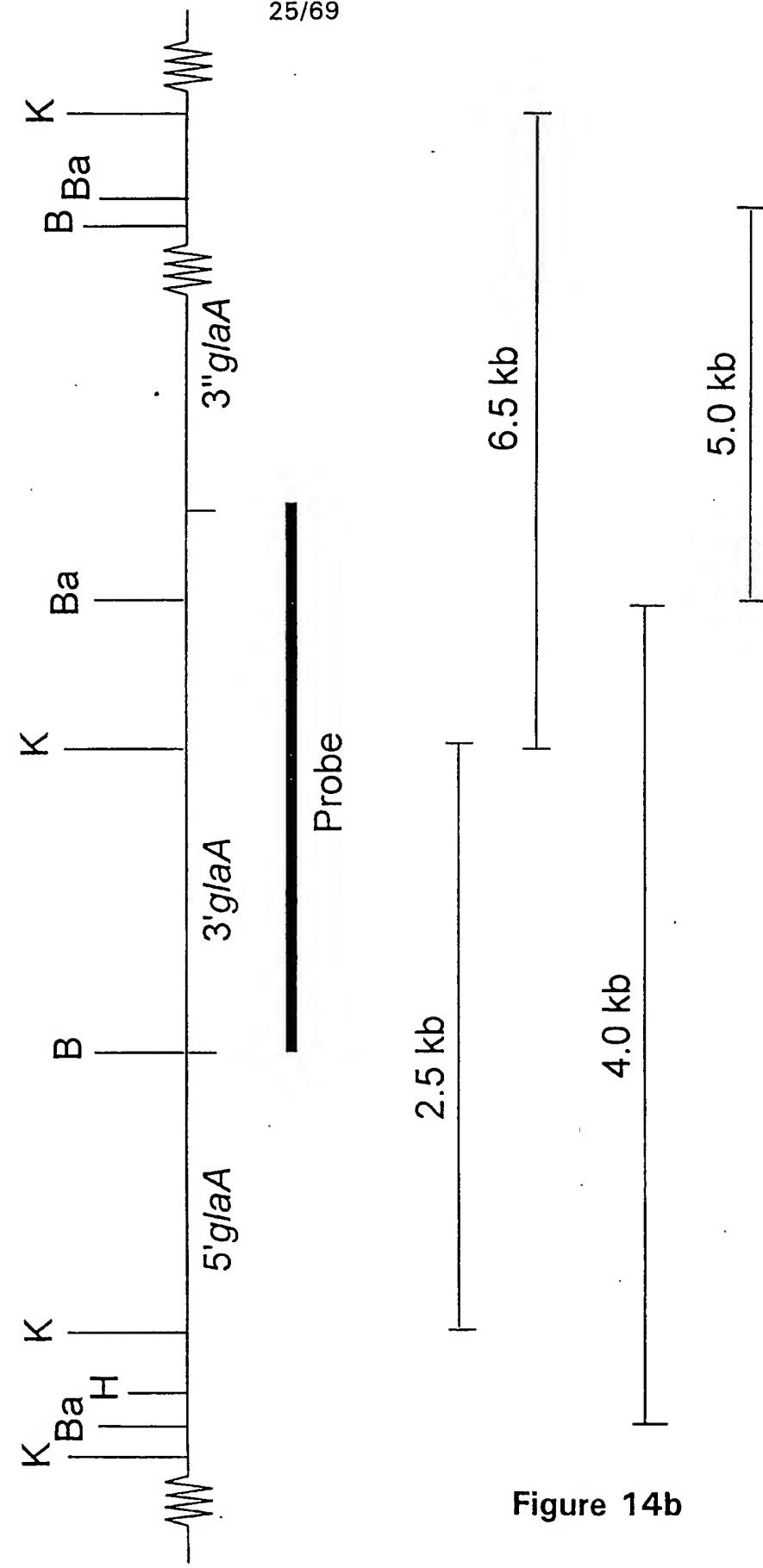


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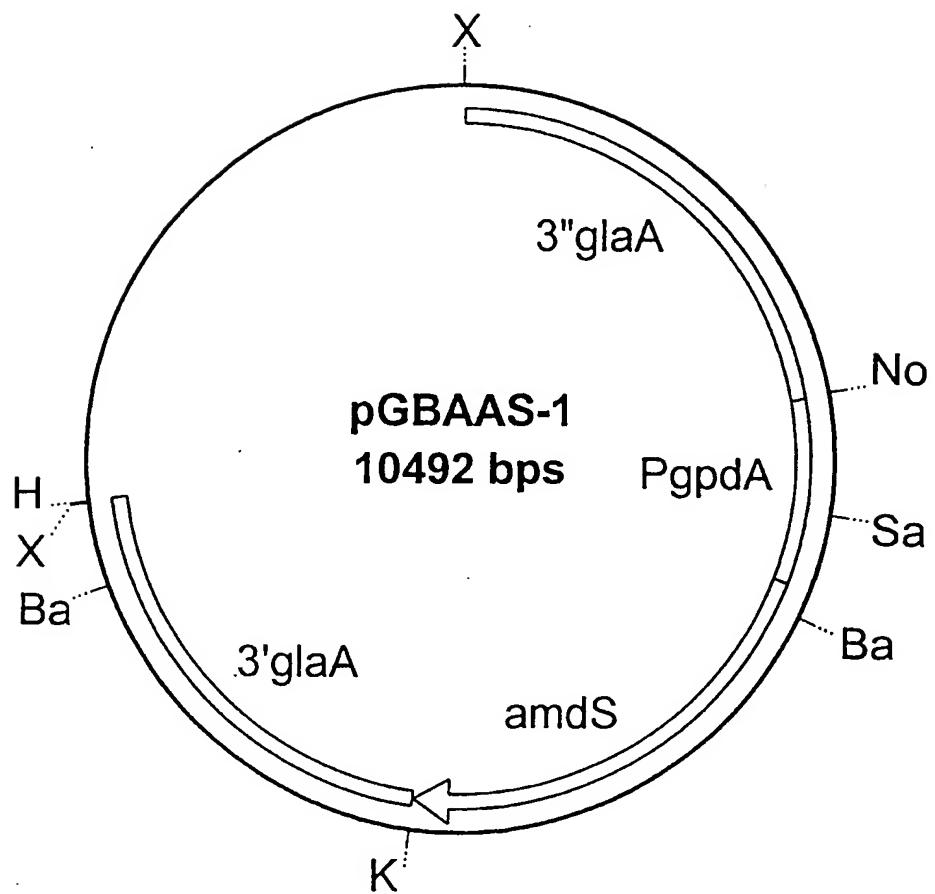


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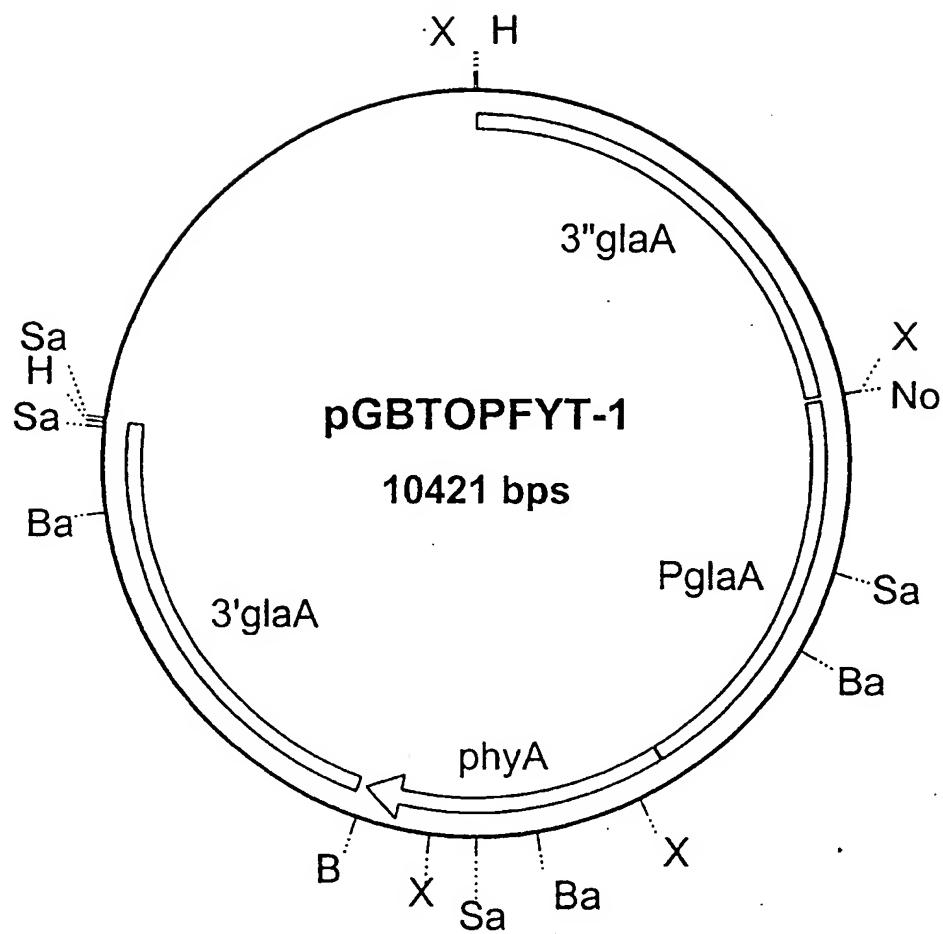


Figure 16

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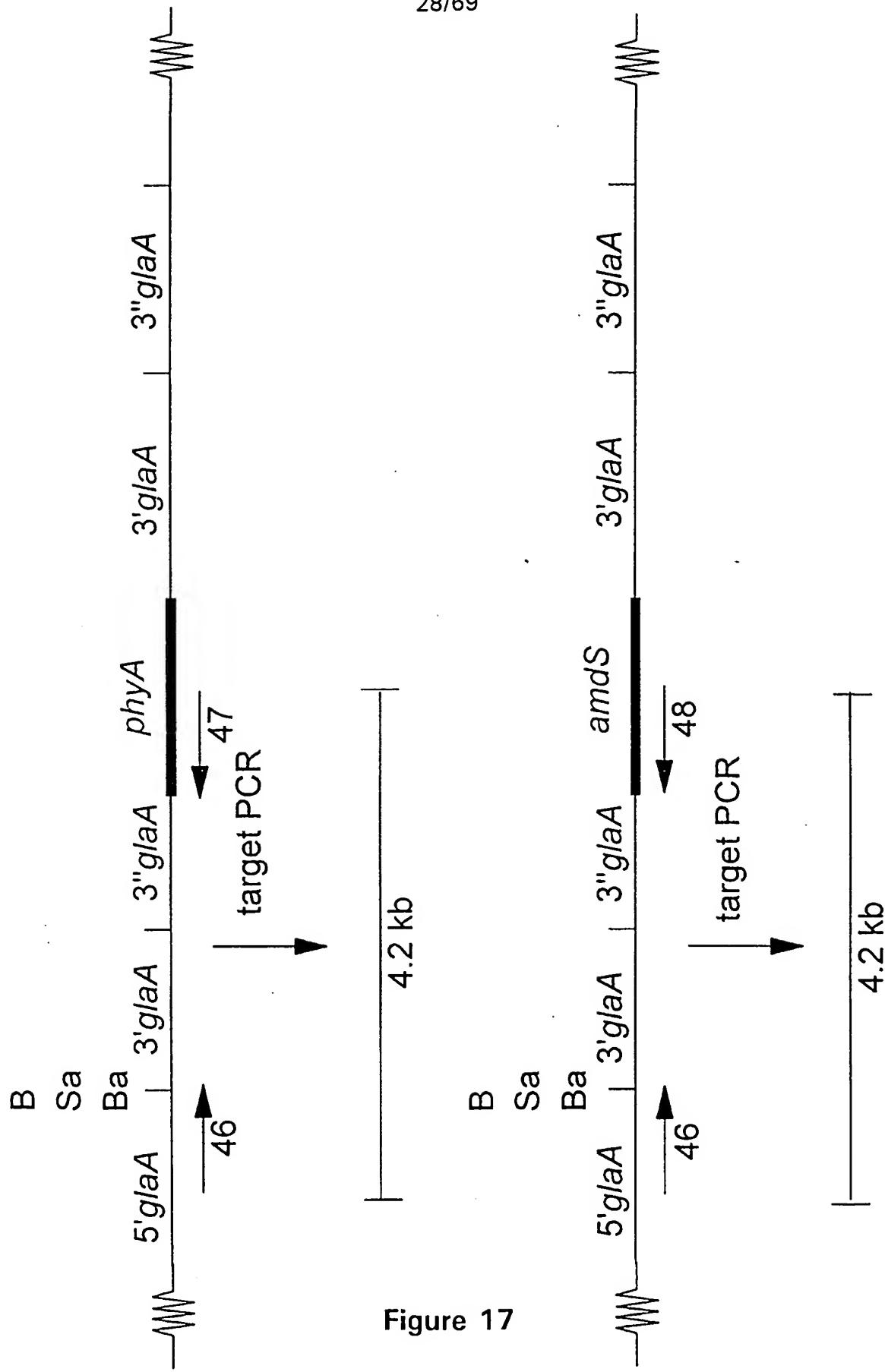


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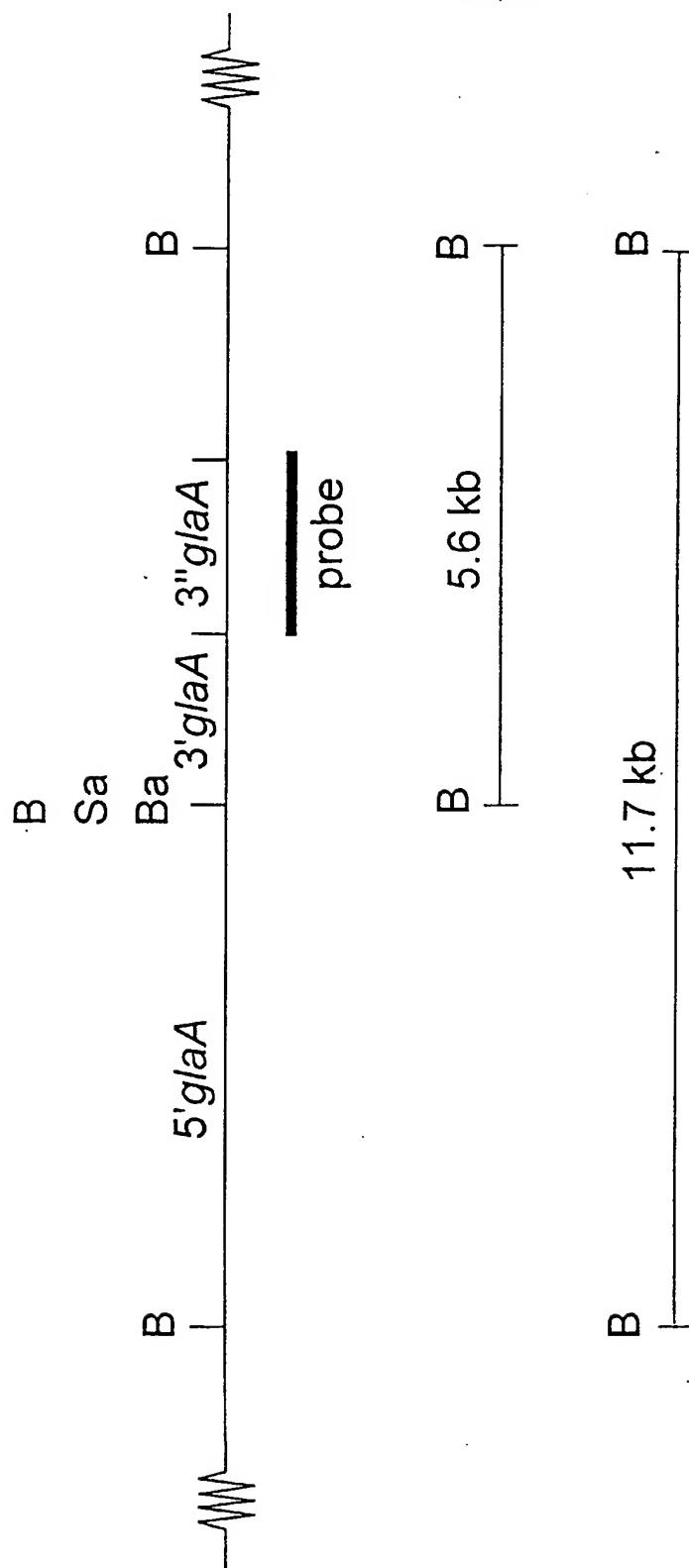


Figure 18

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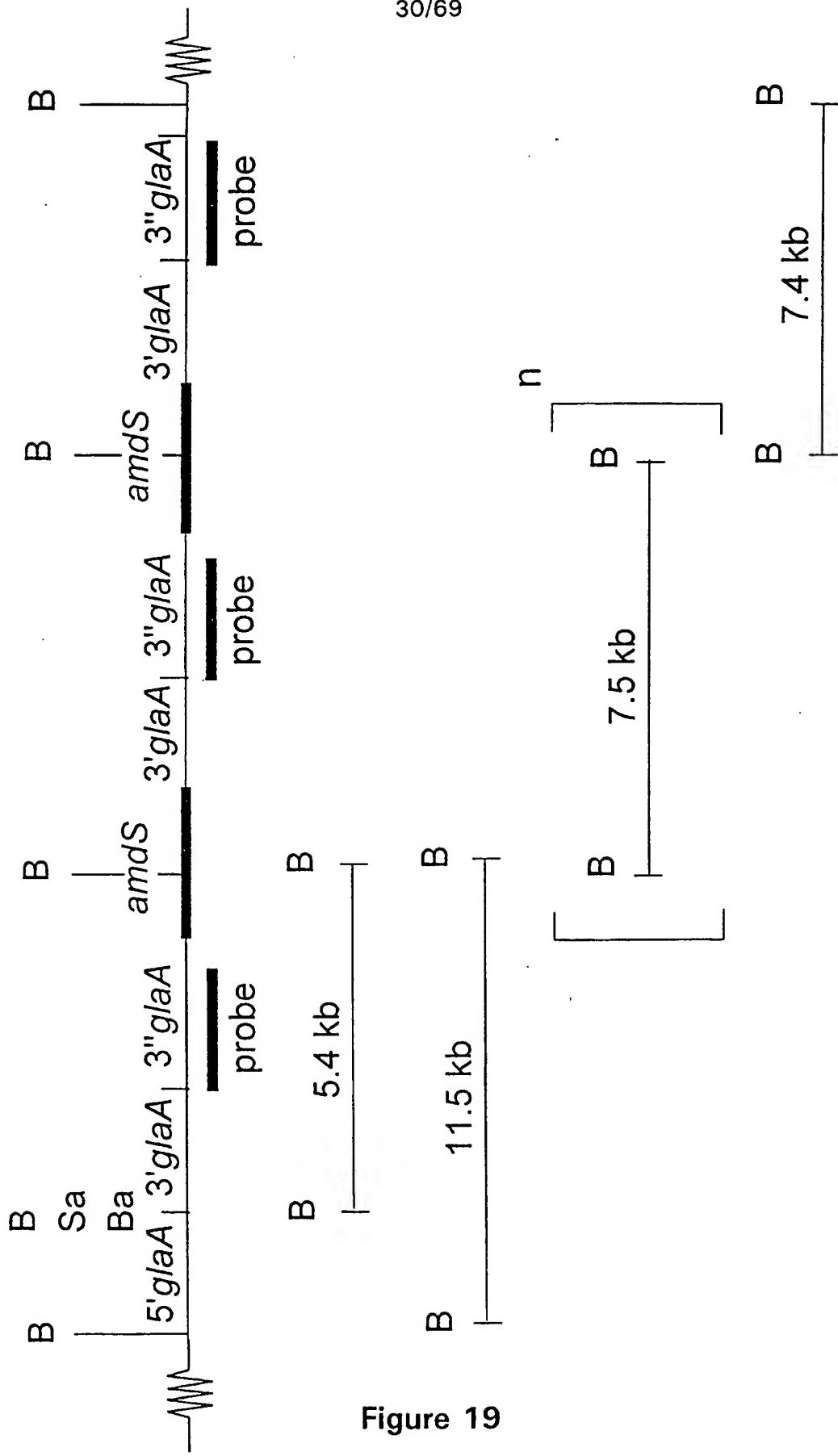


Figure 19

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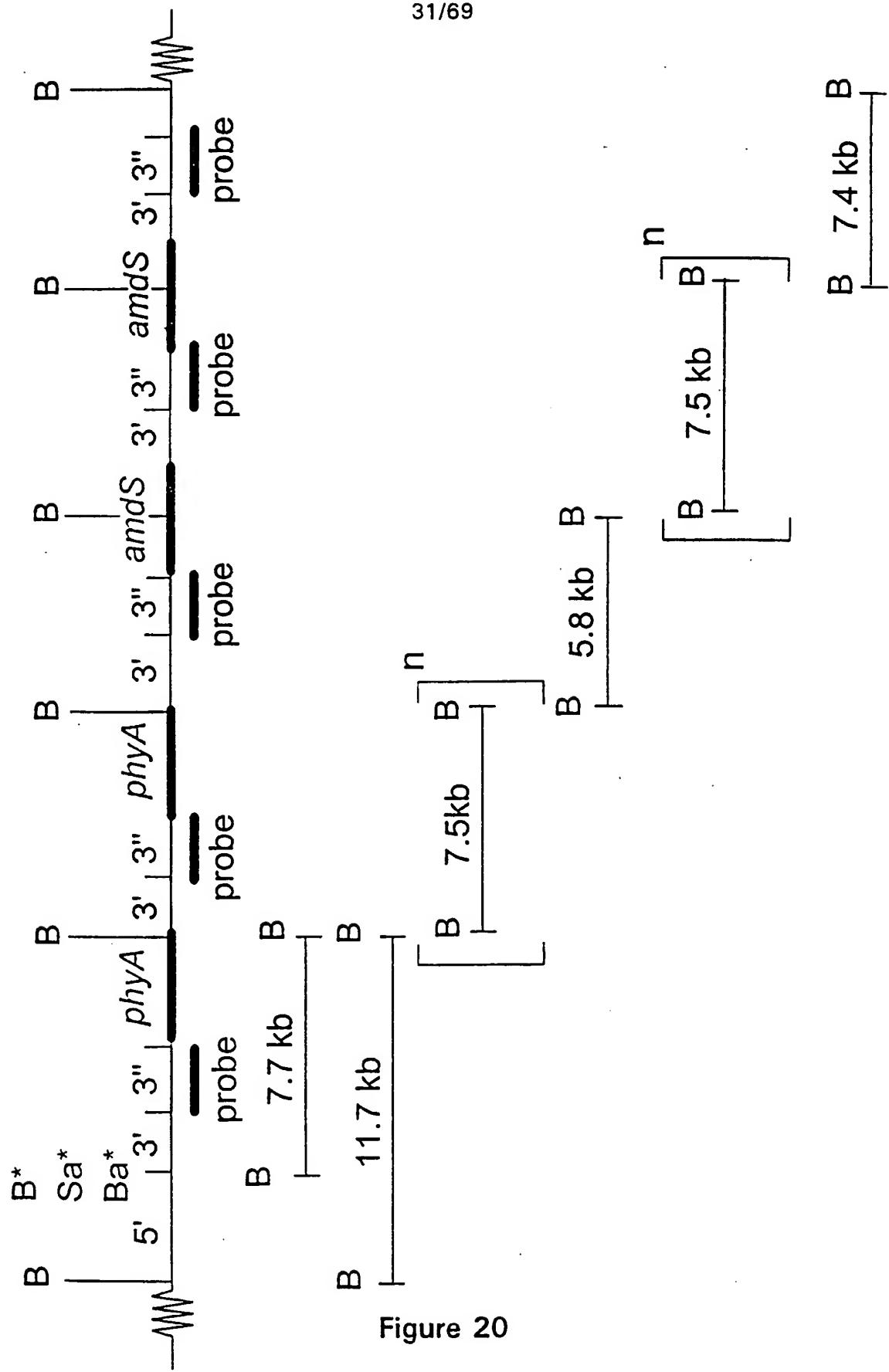


Figure 20

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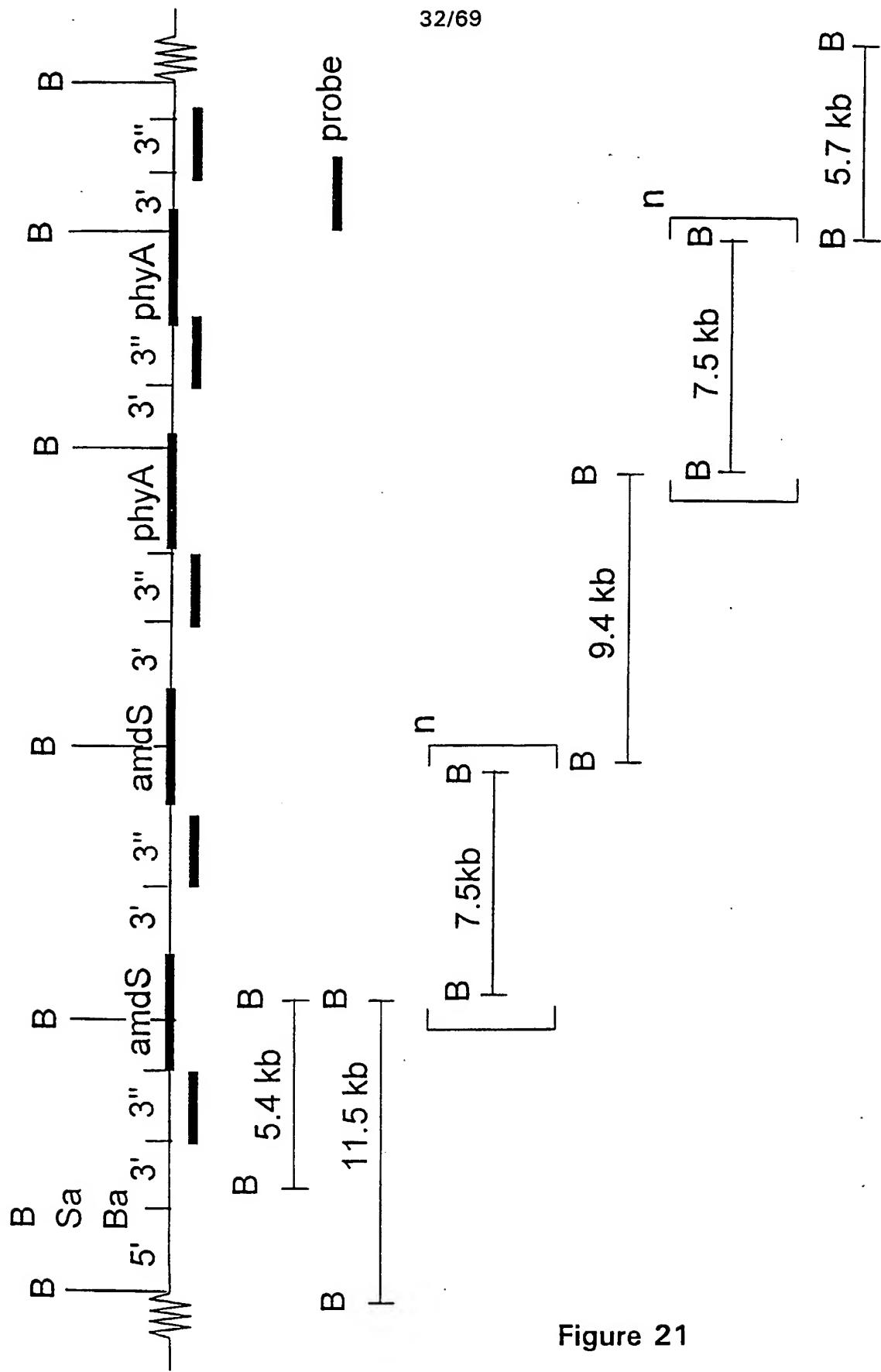


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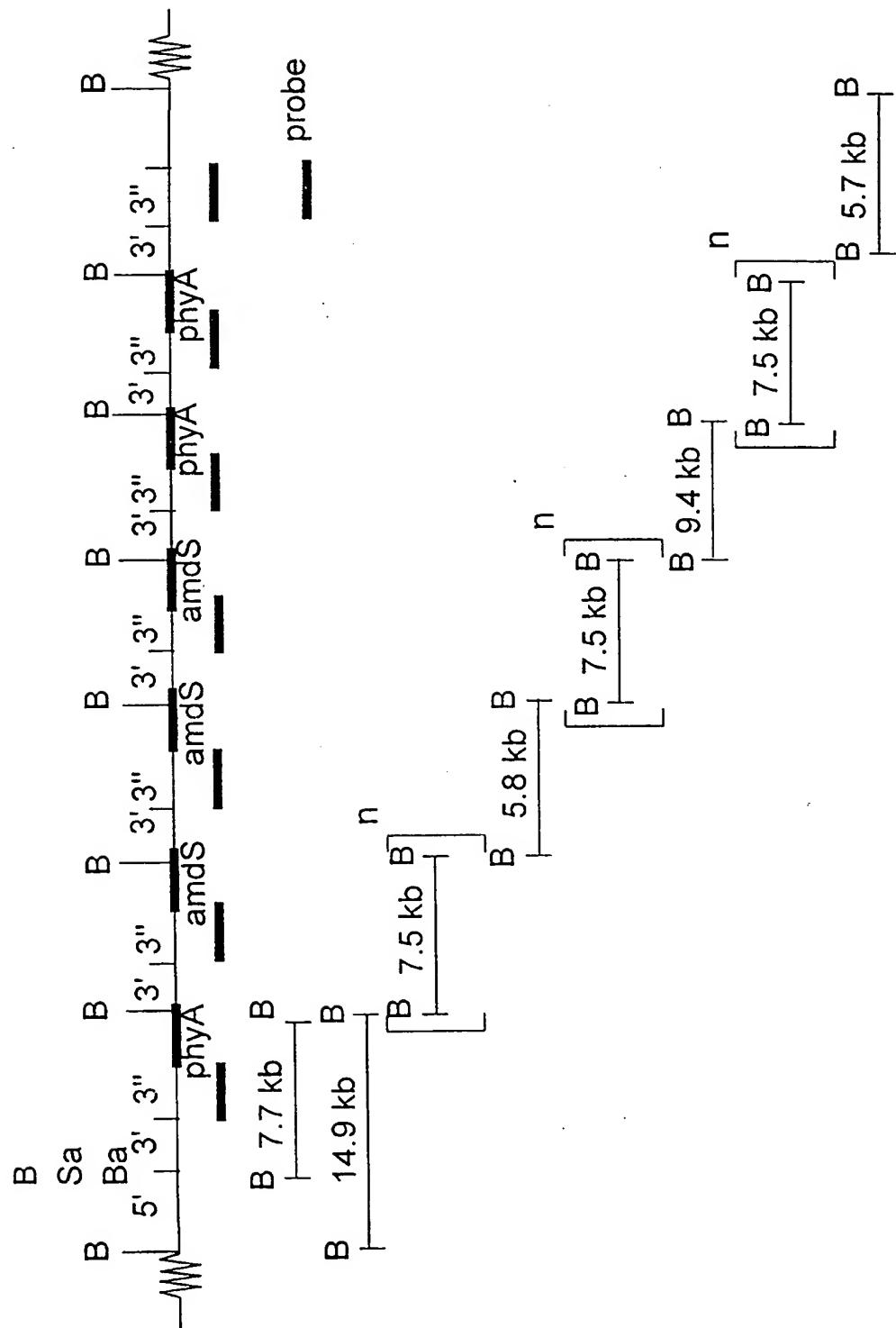


Figure 22

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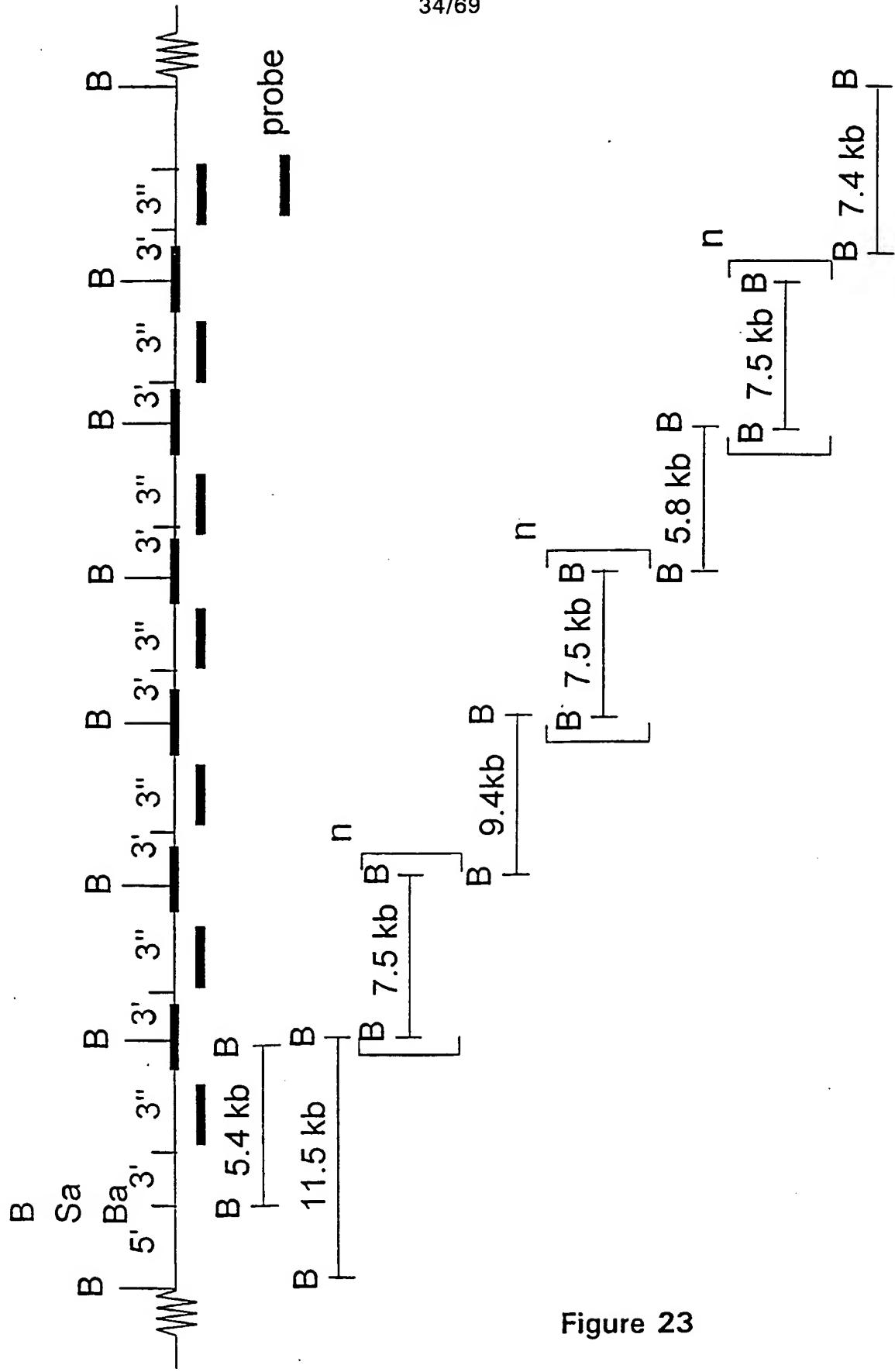


Figure 23

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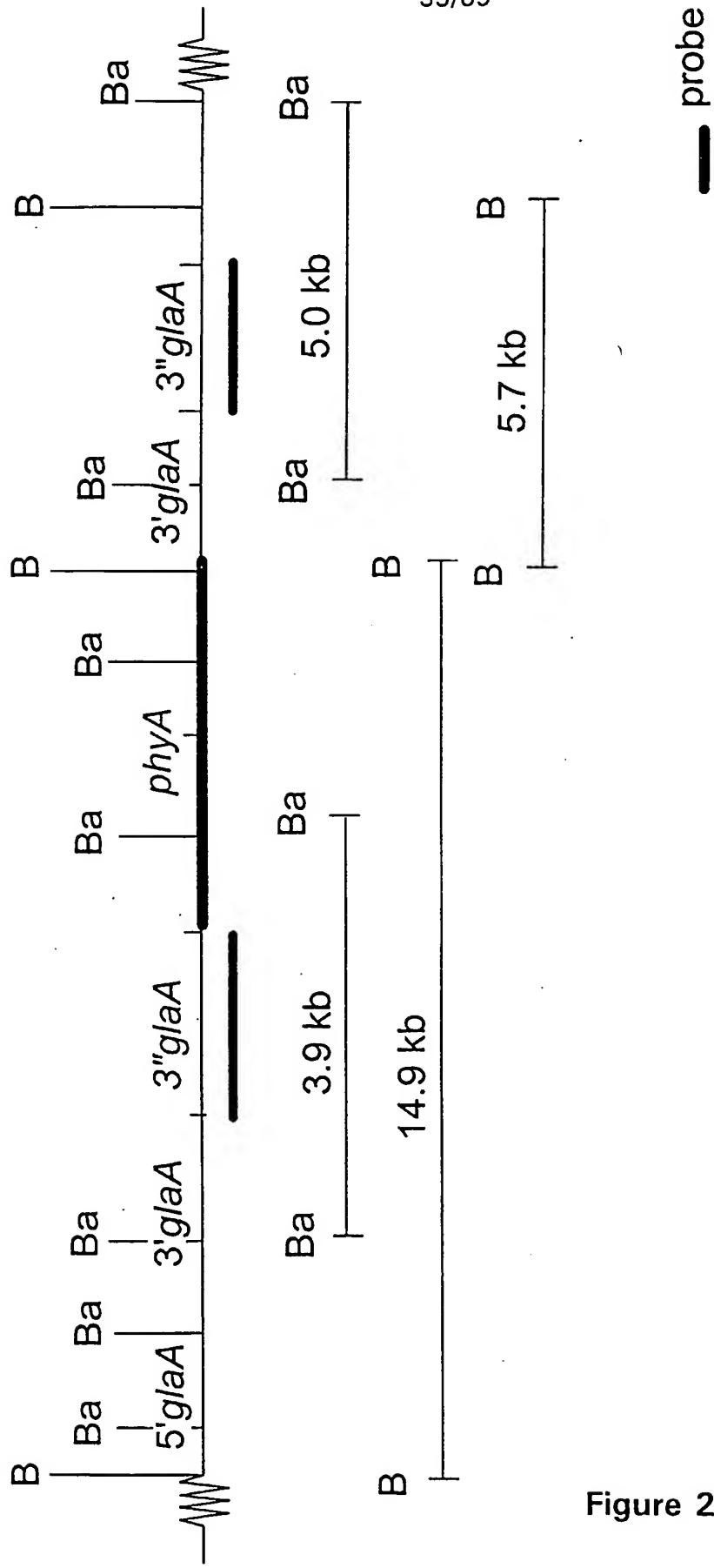


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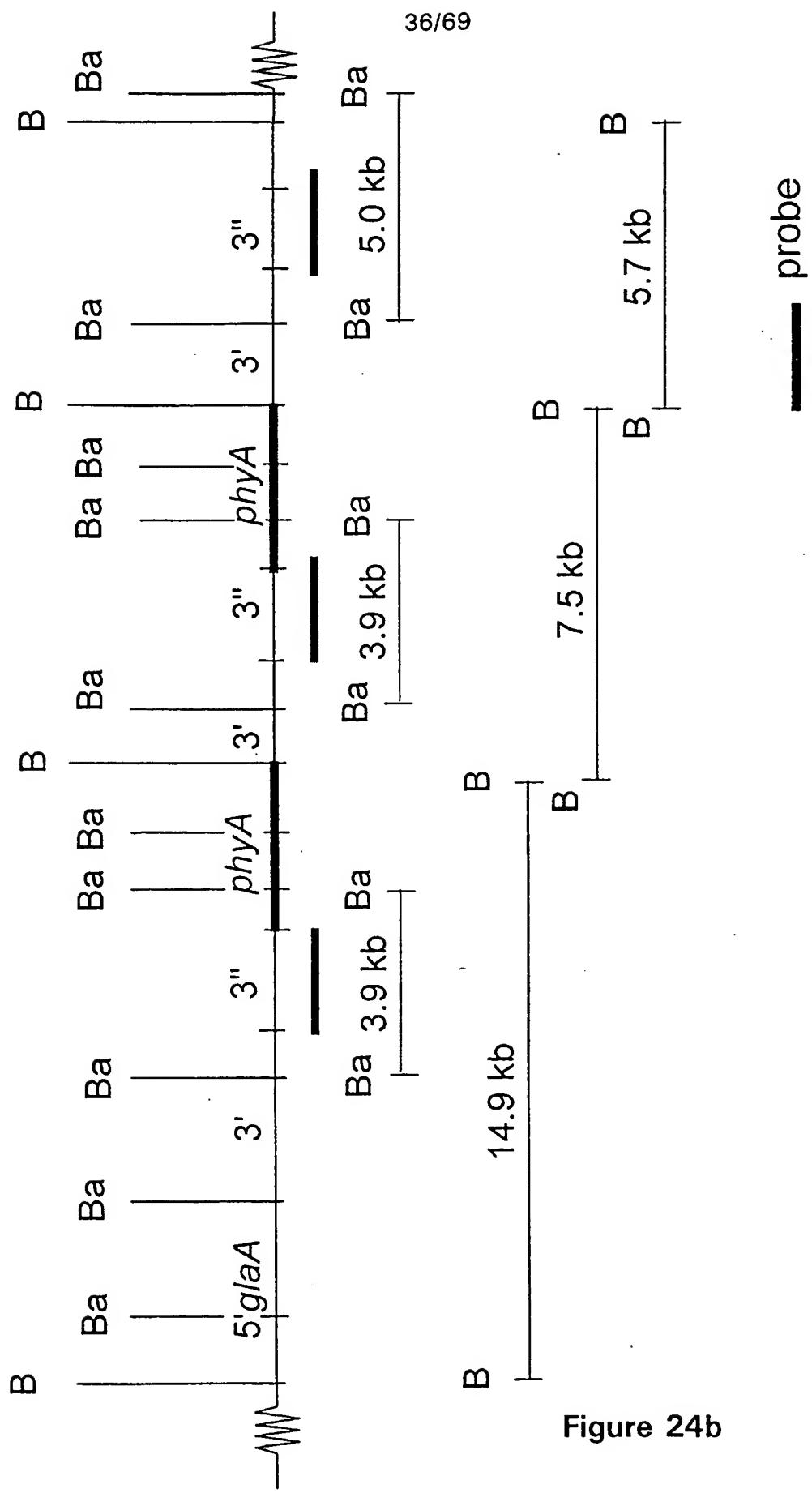


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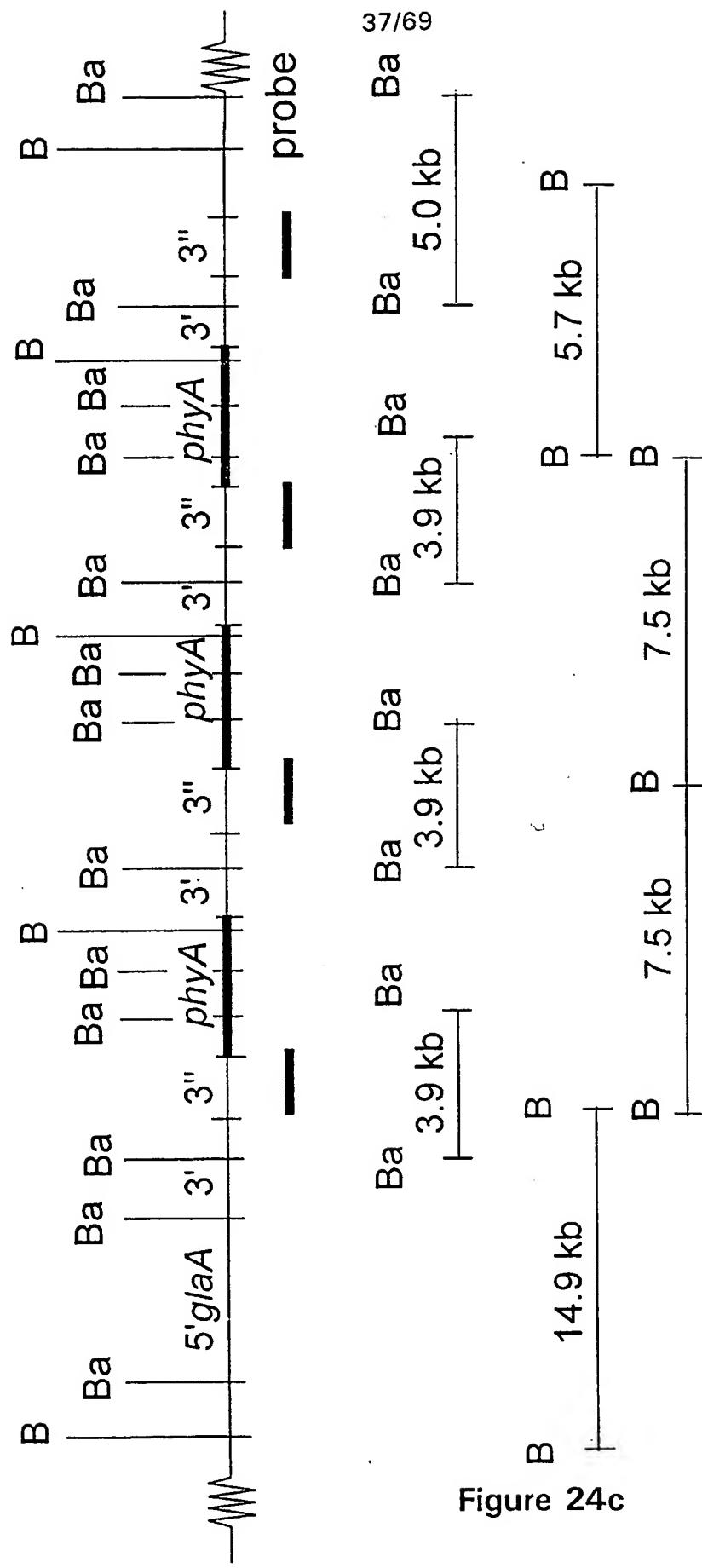


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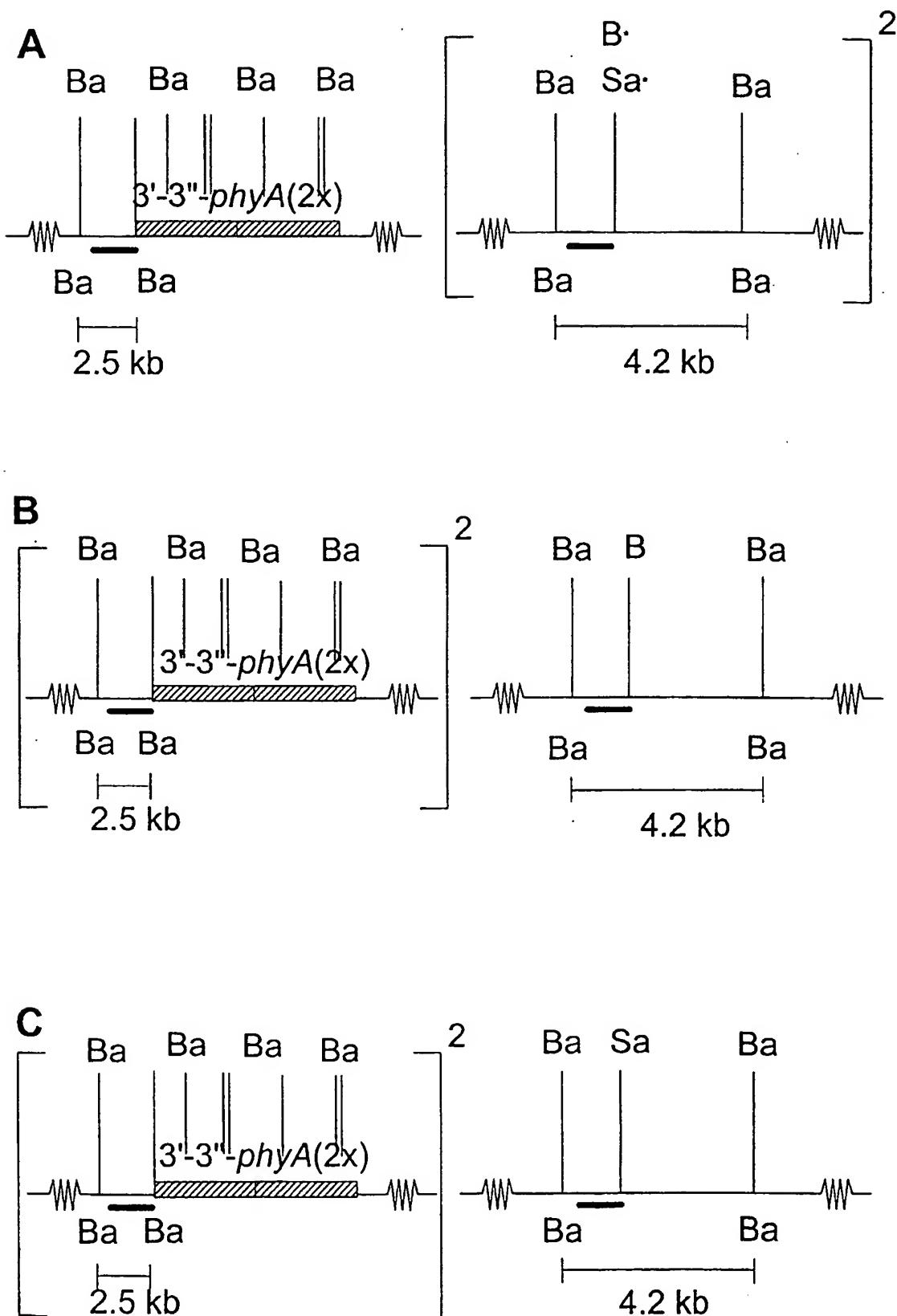


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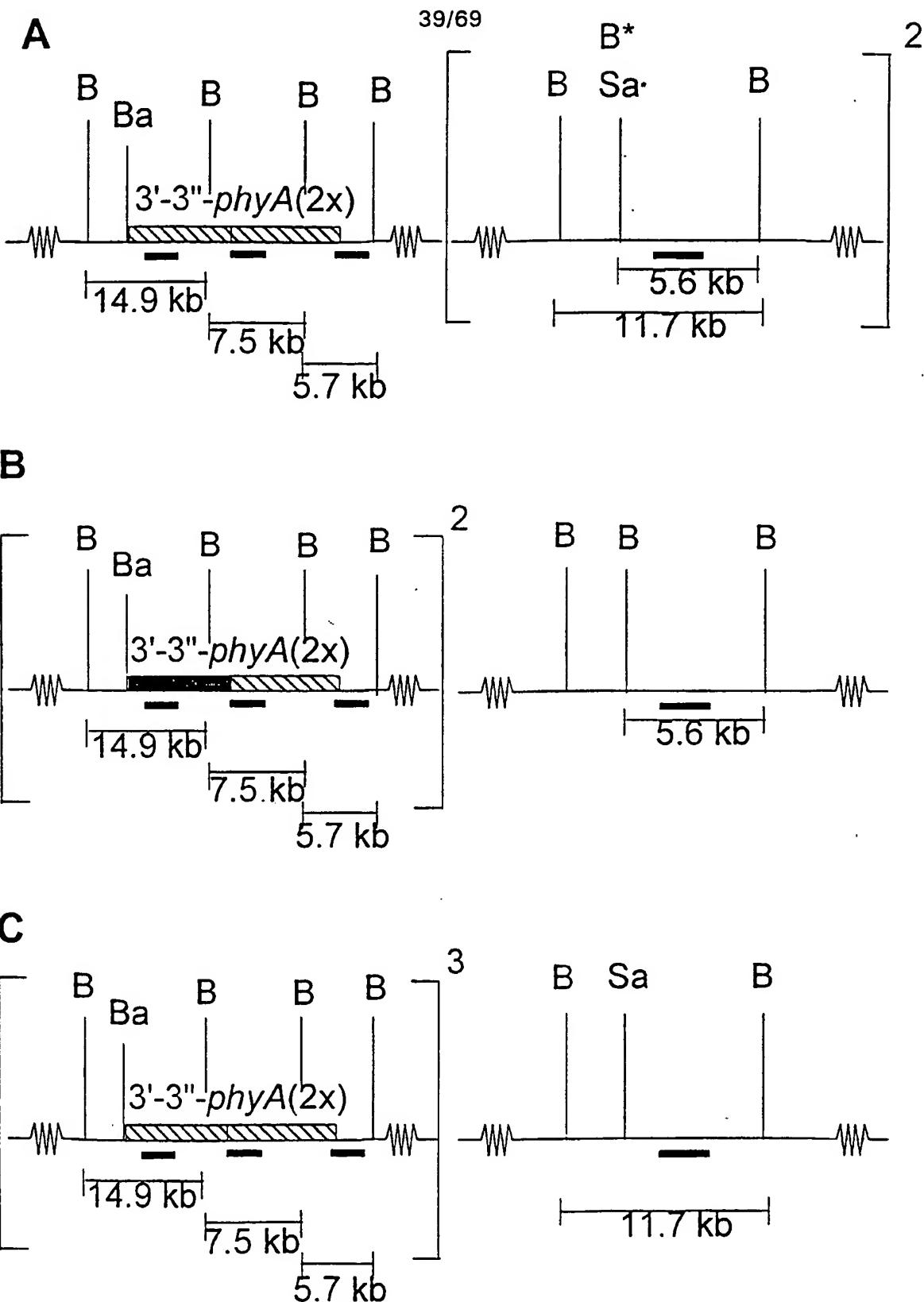


Figure 26

— probe

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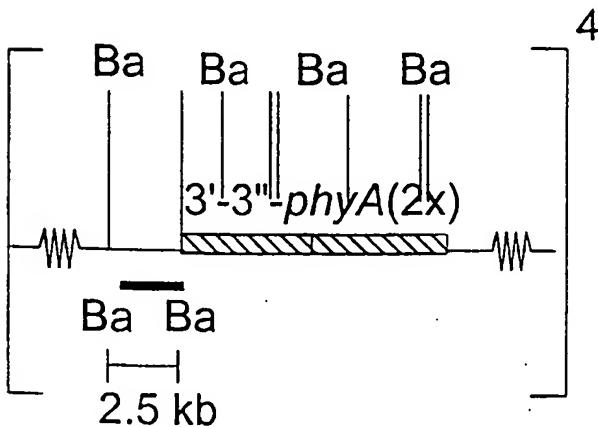
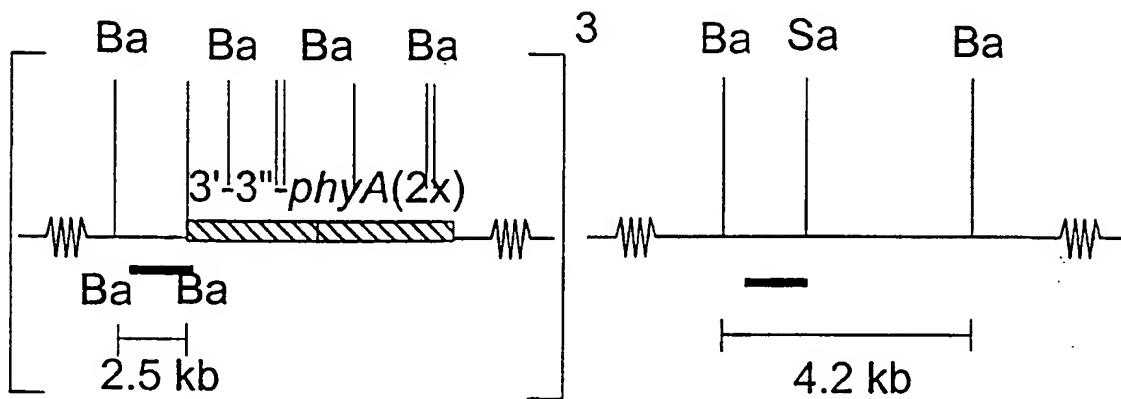
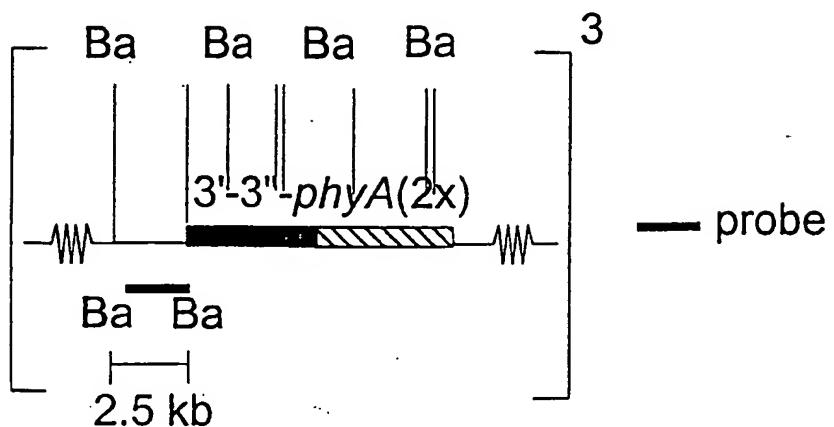


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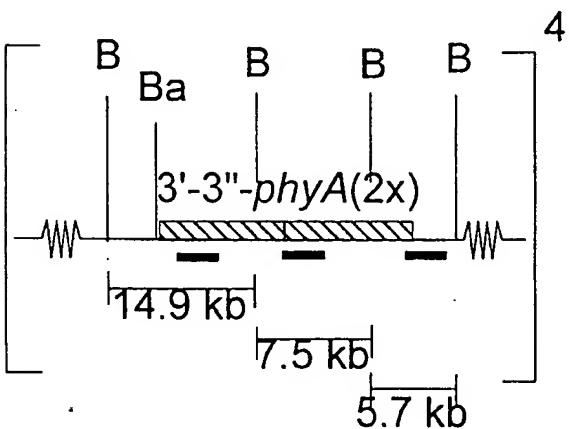
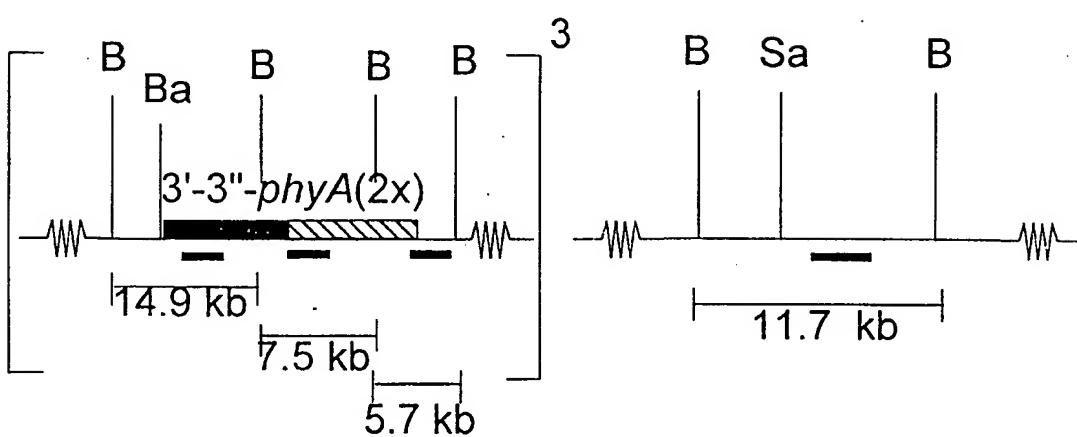
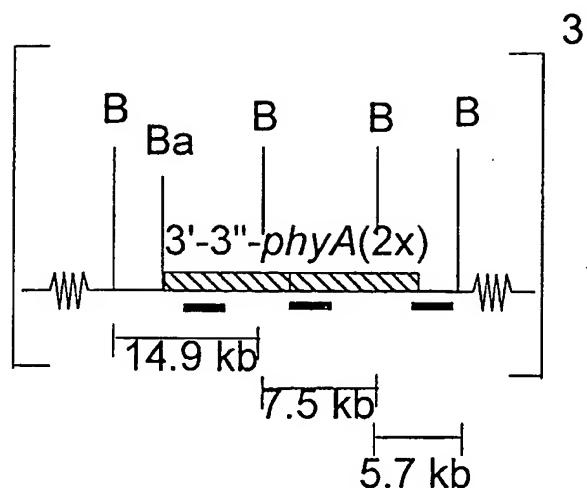


Figure 28

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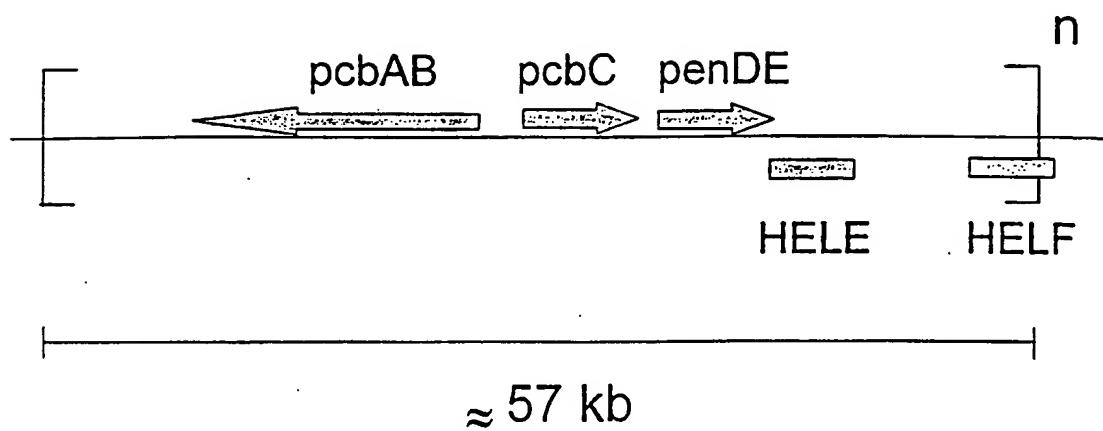


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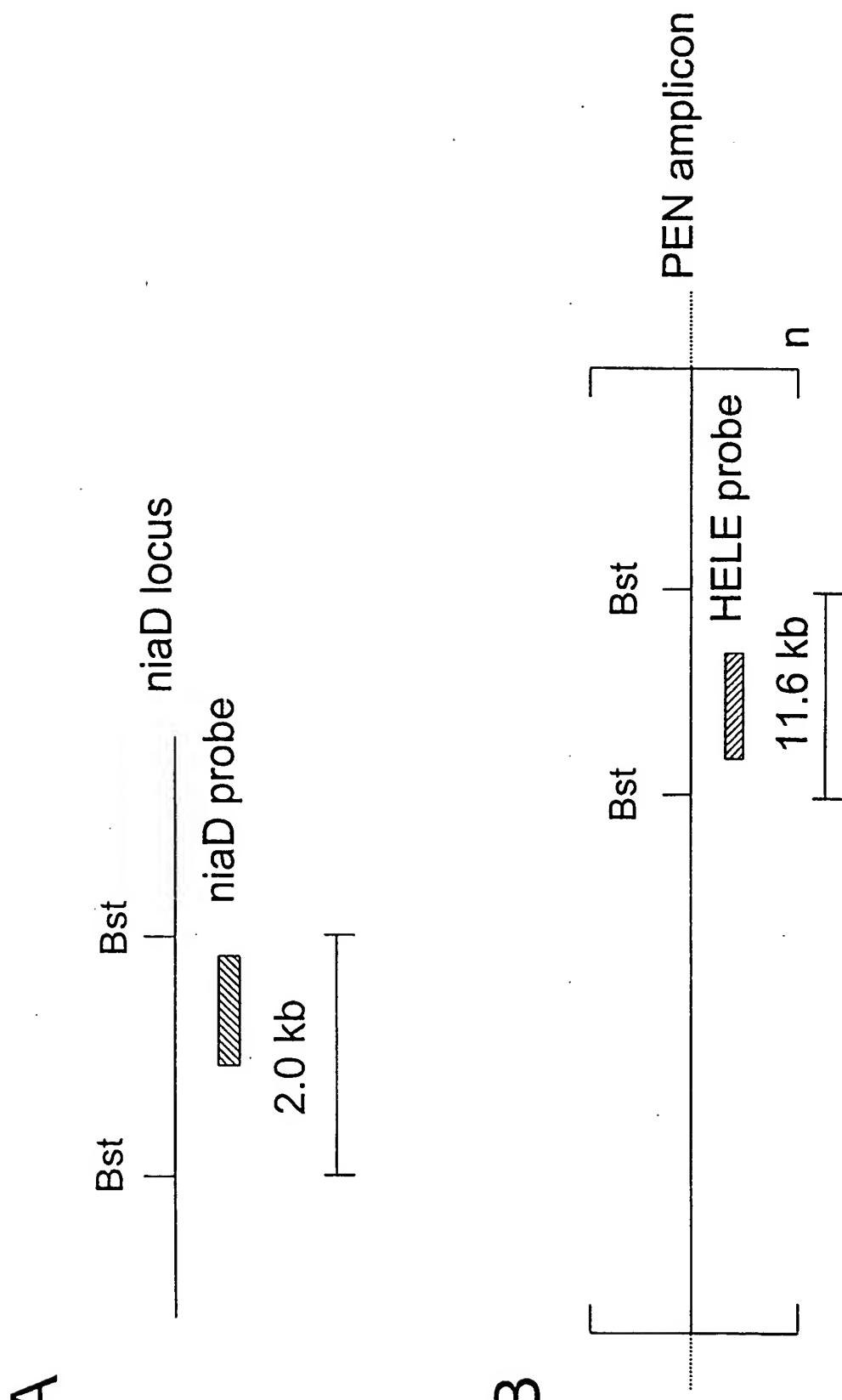


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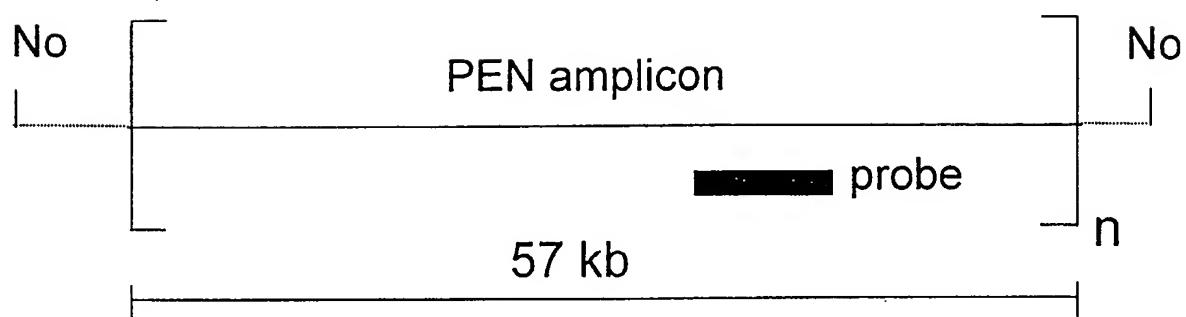


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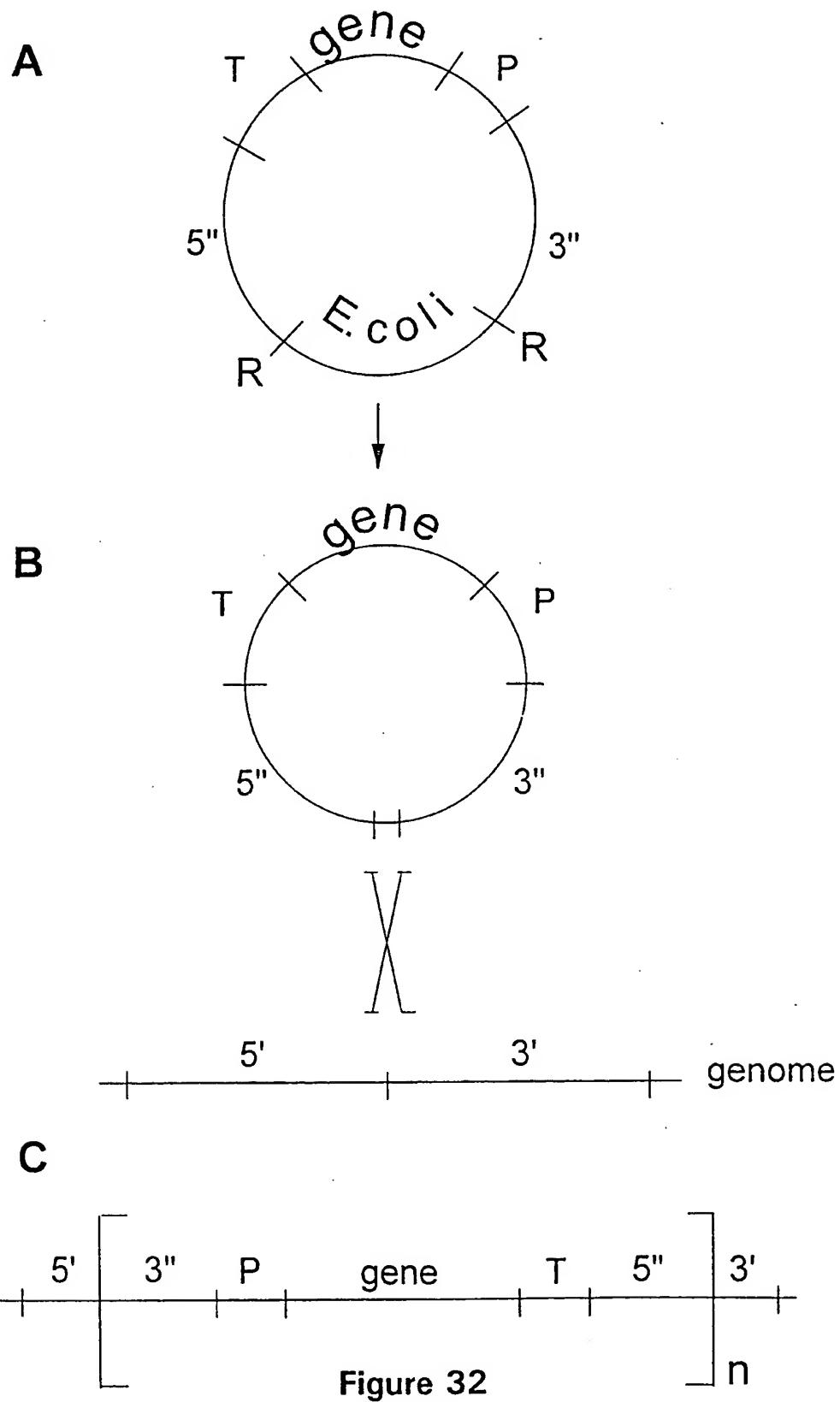


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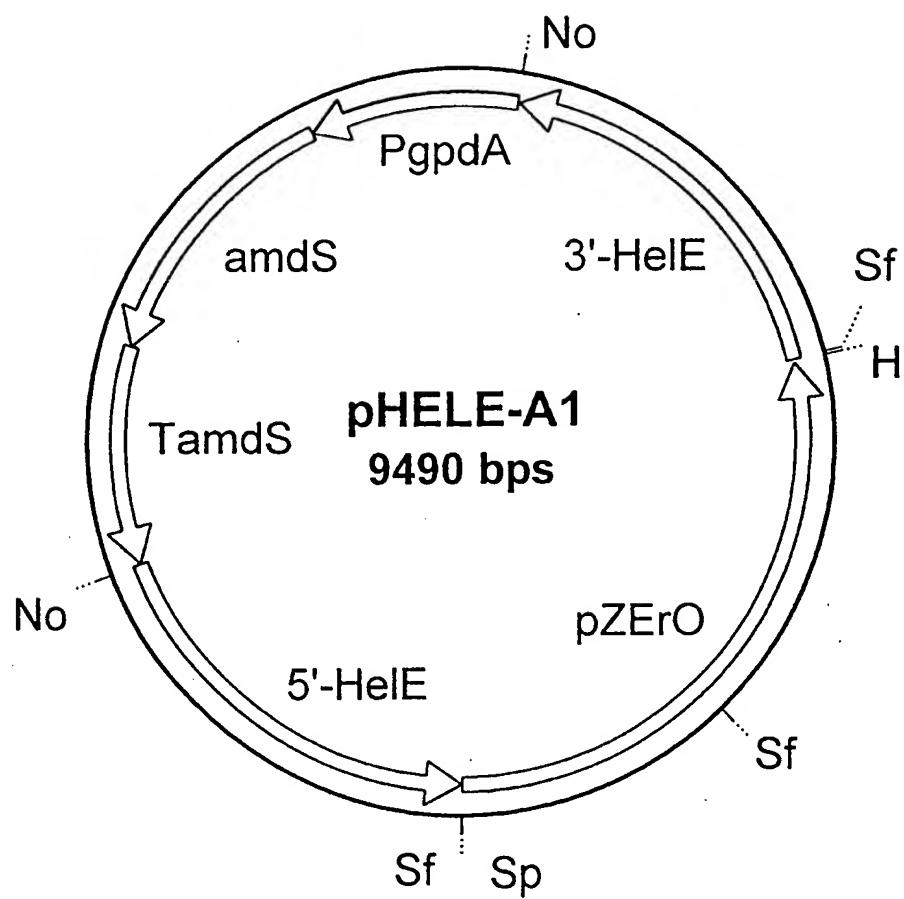


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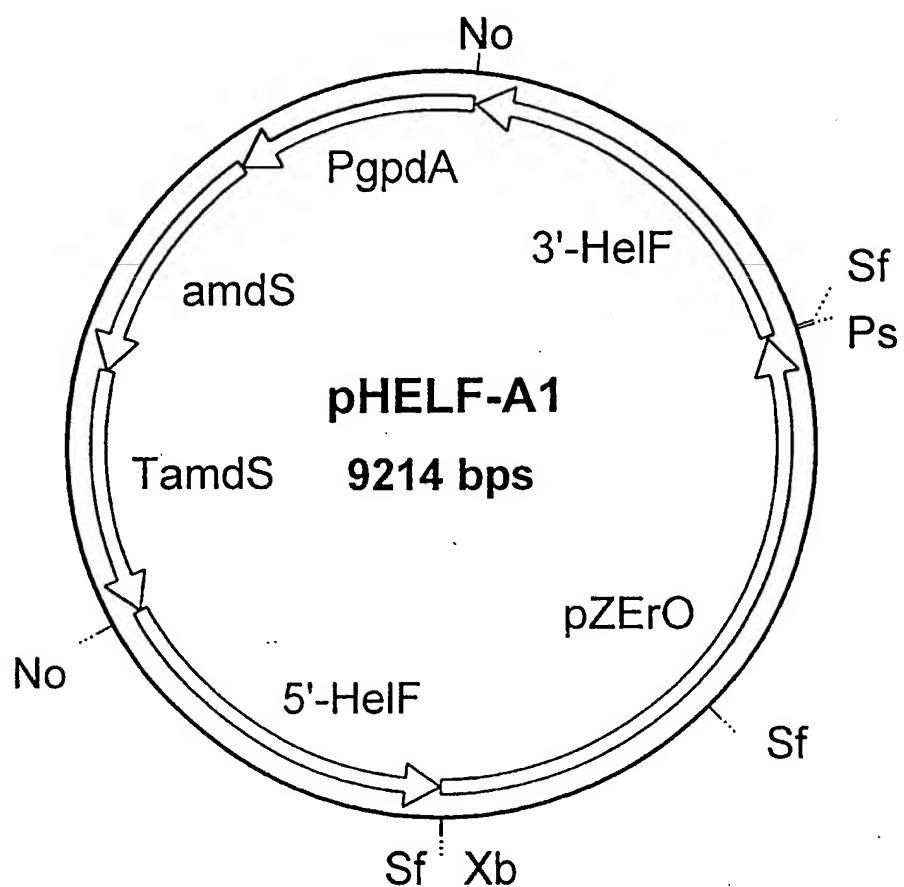


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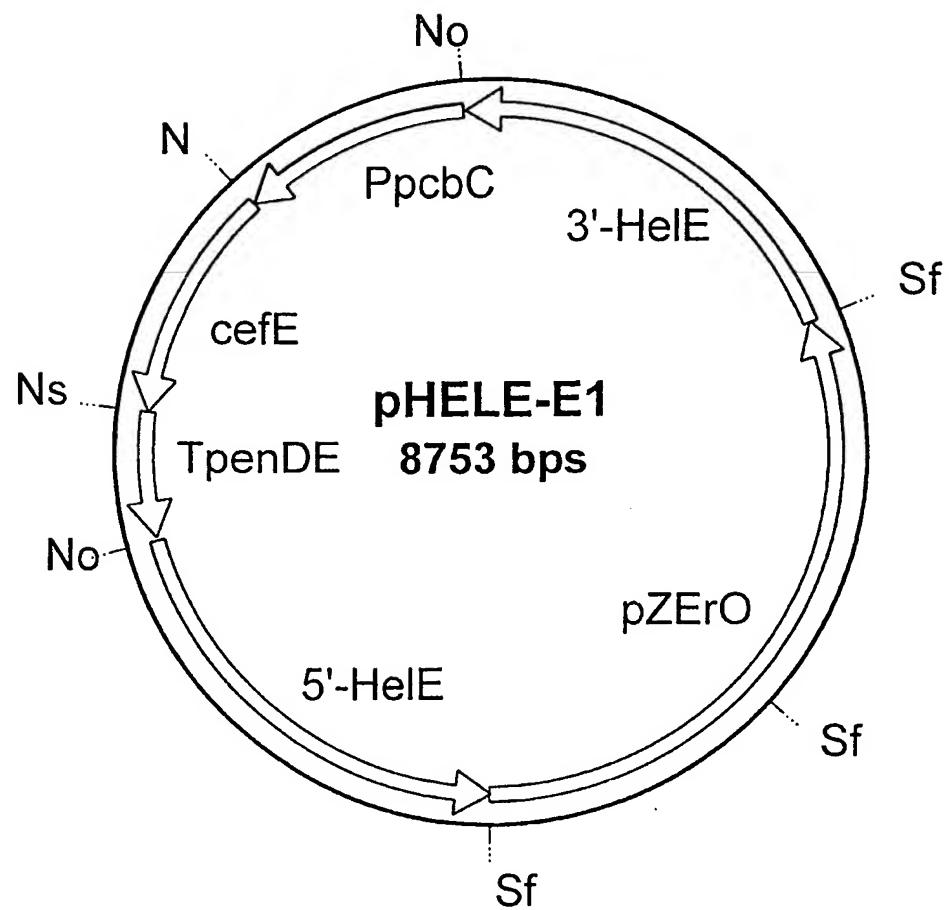


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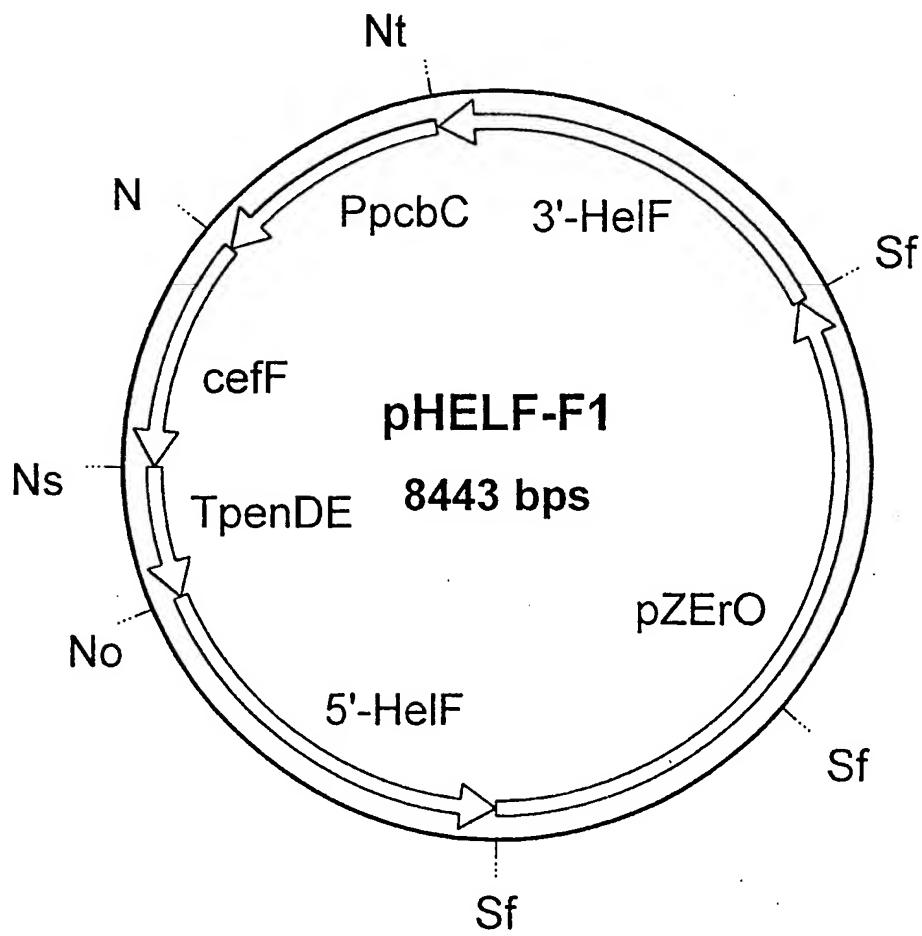


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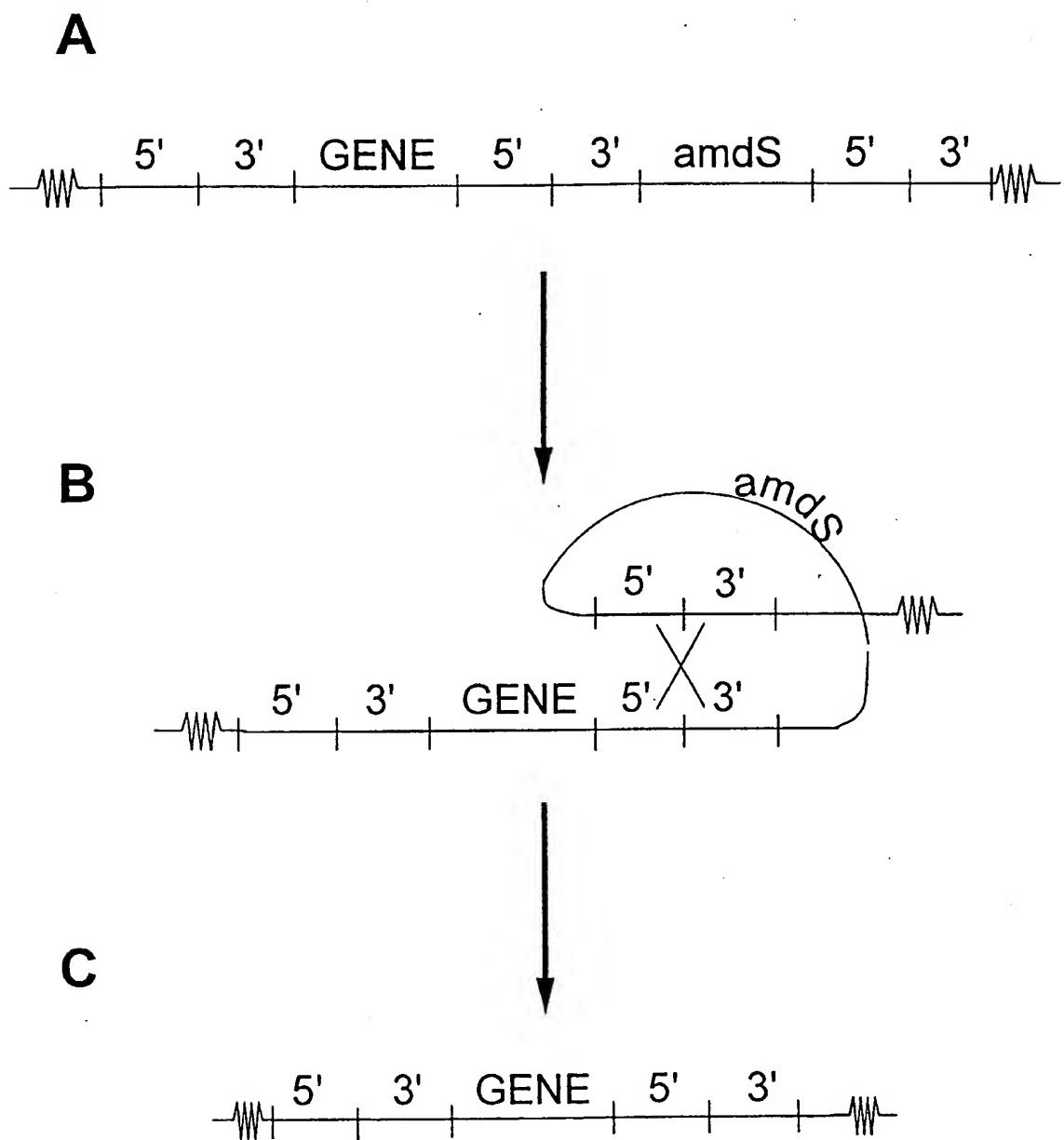


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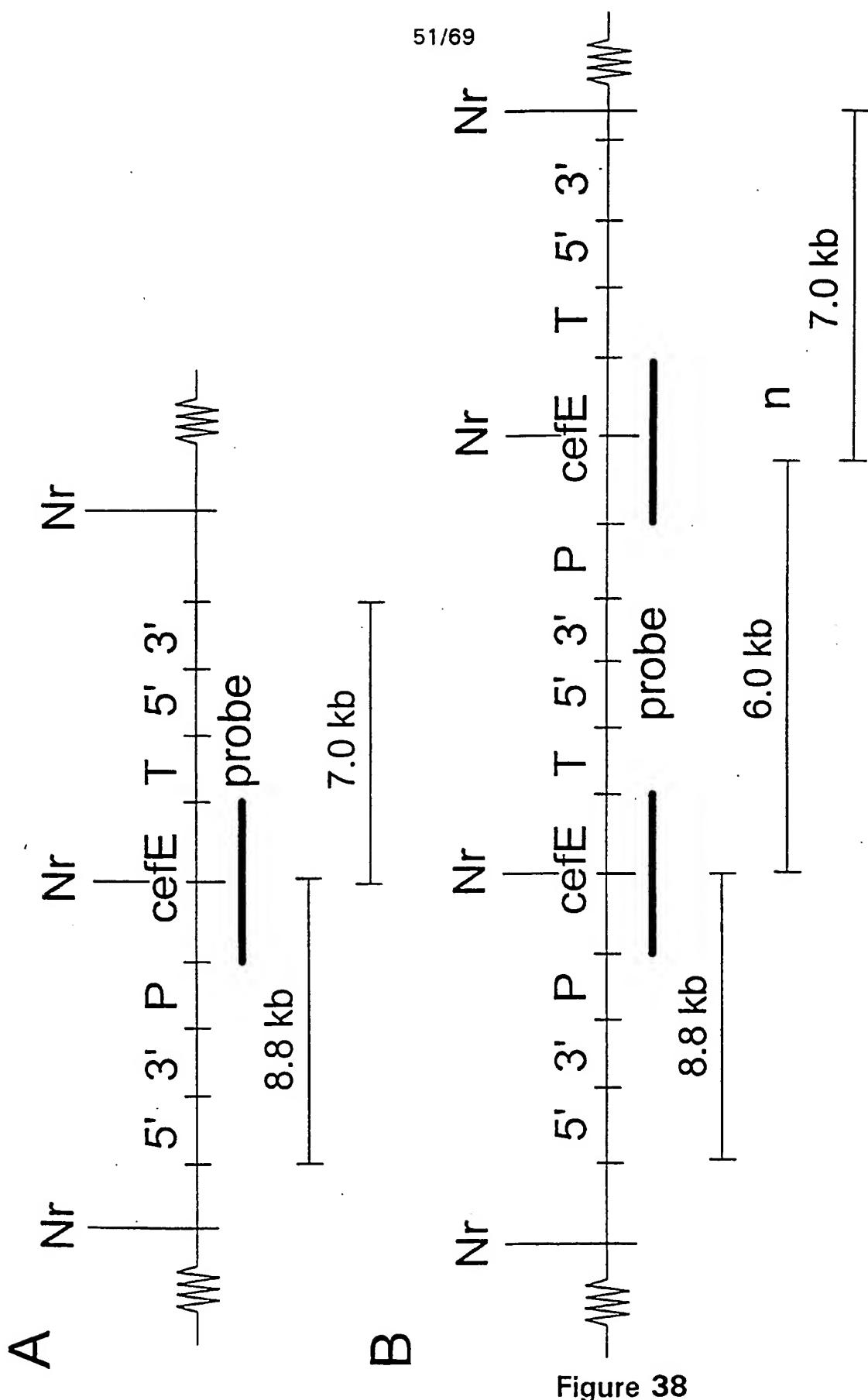


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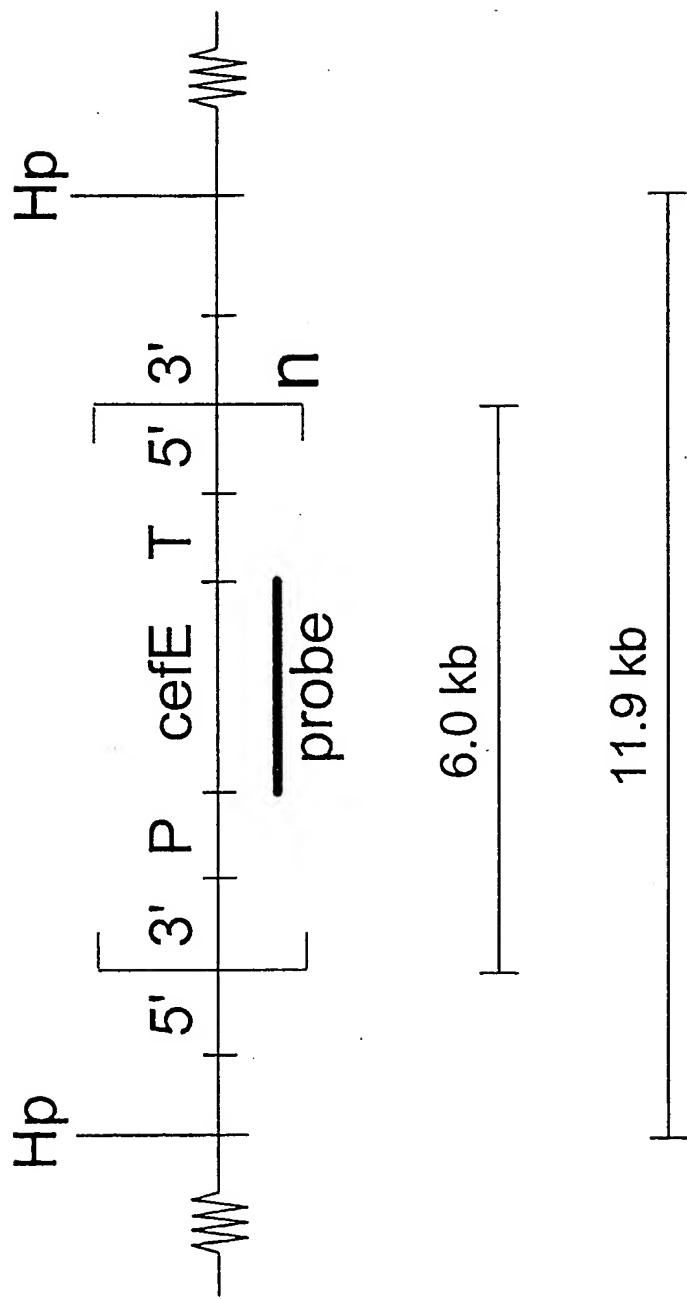
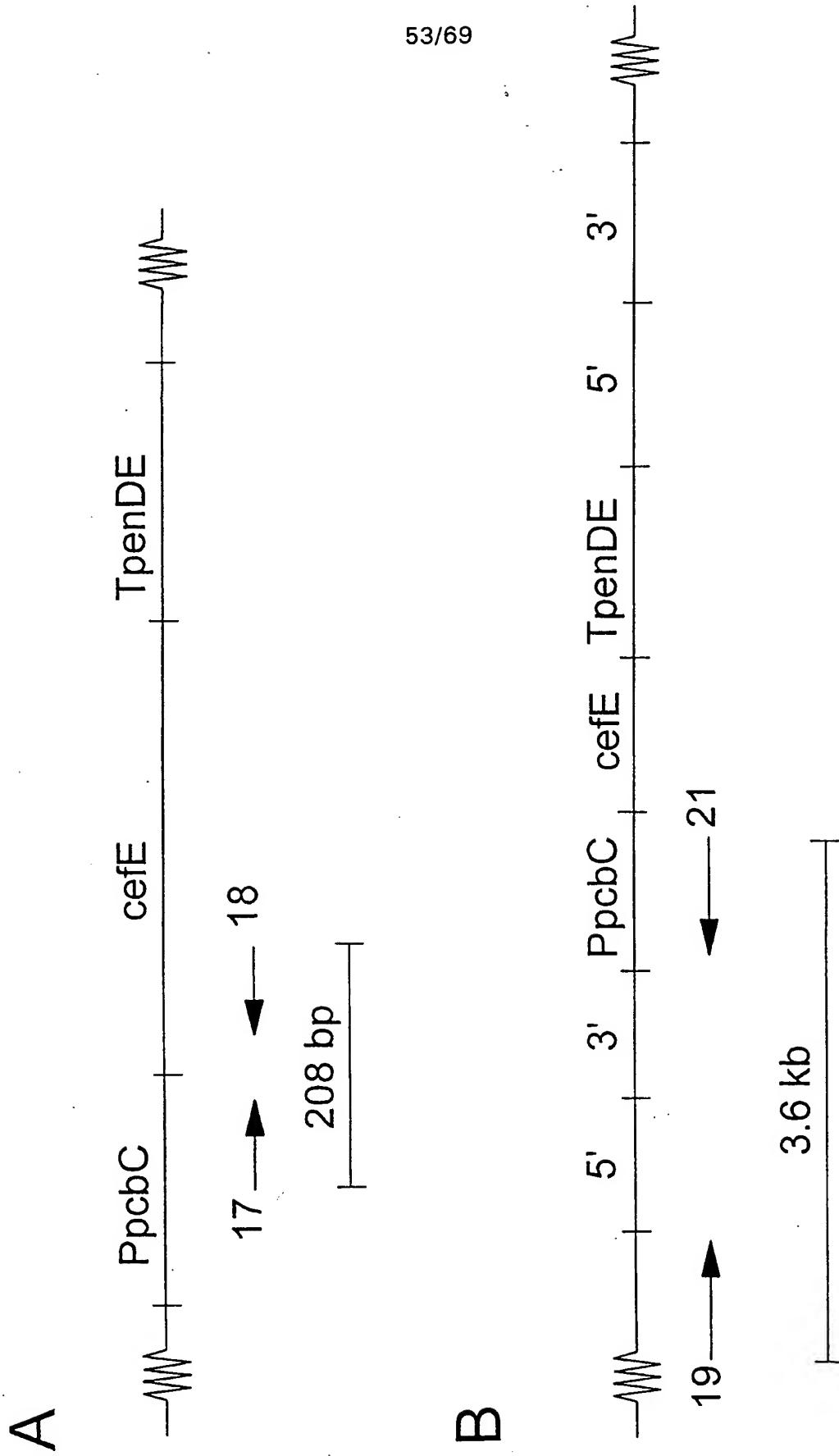
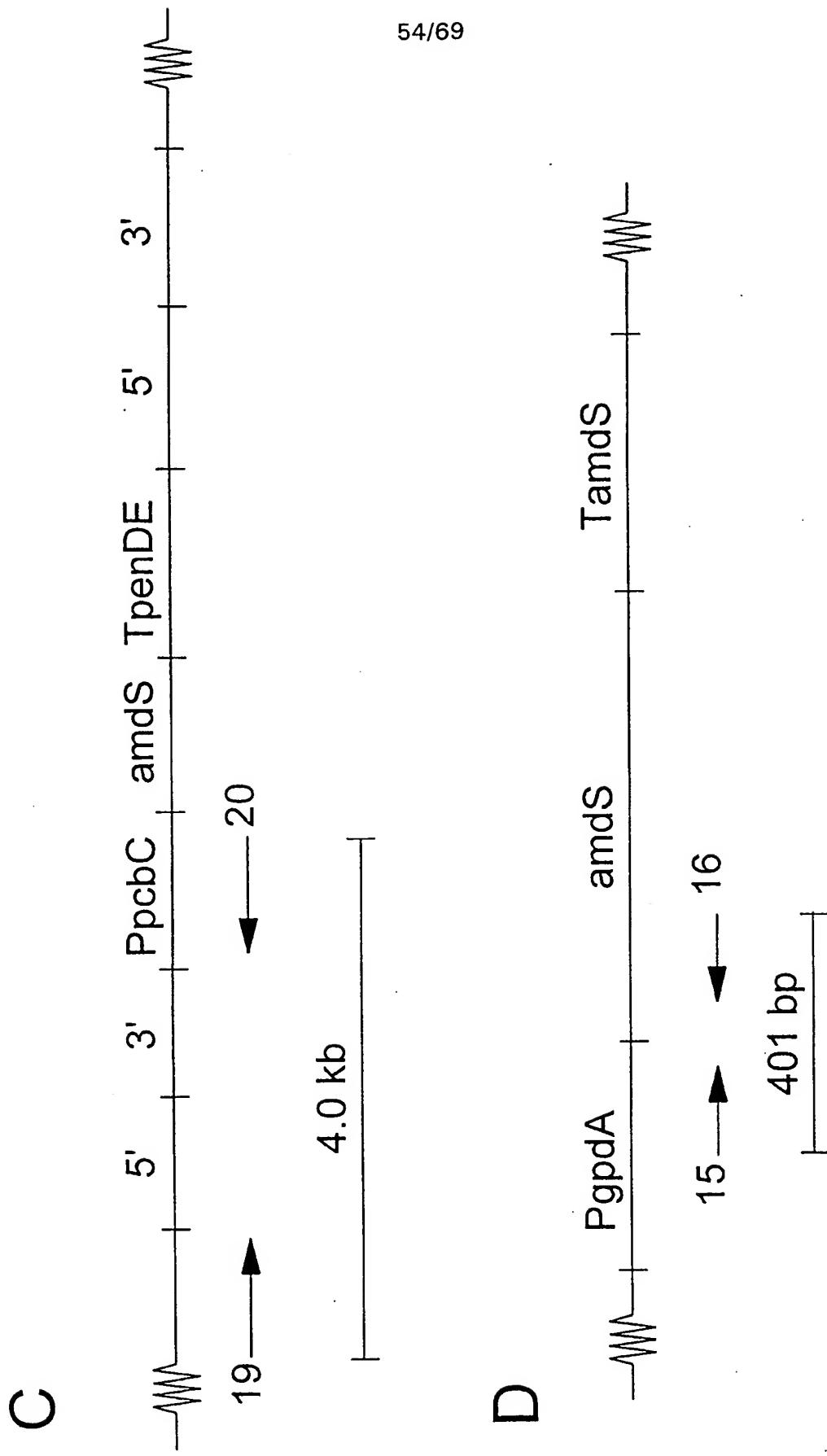


Figure 39

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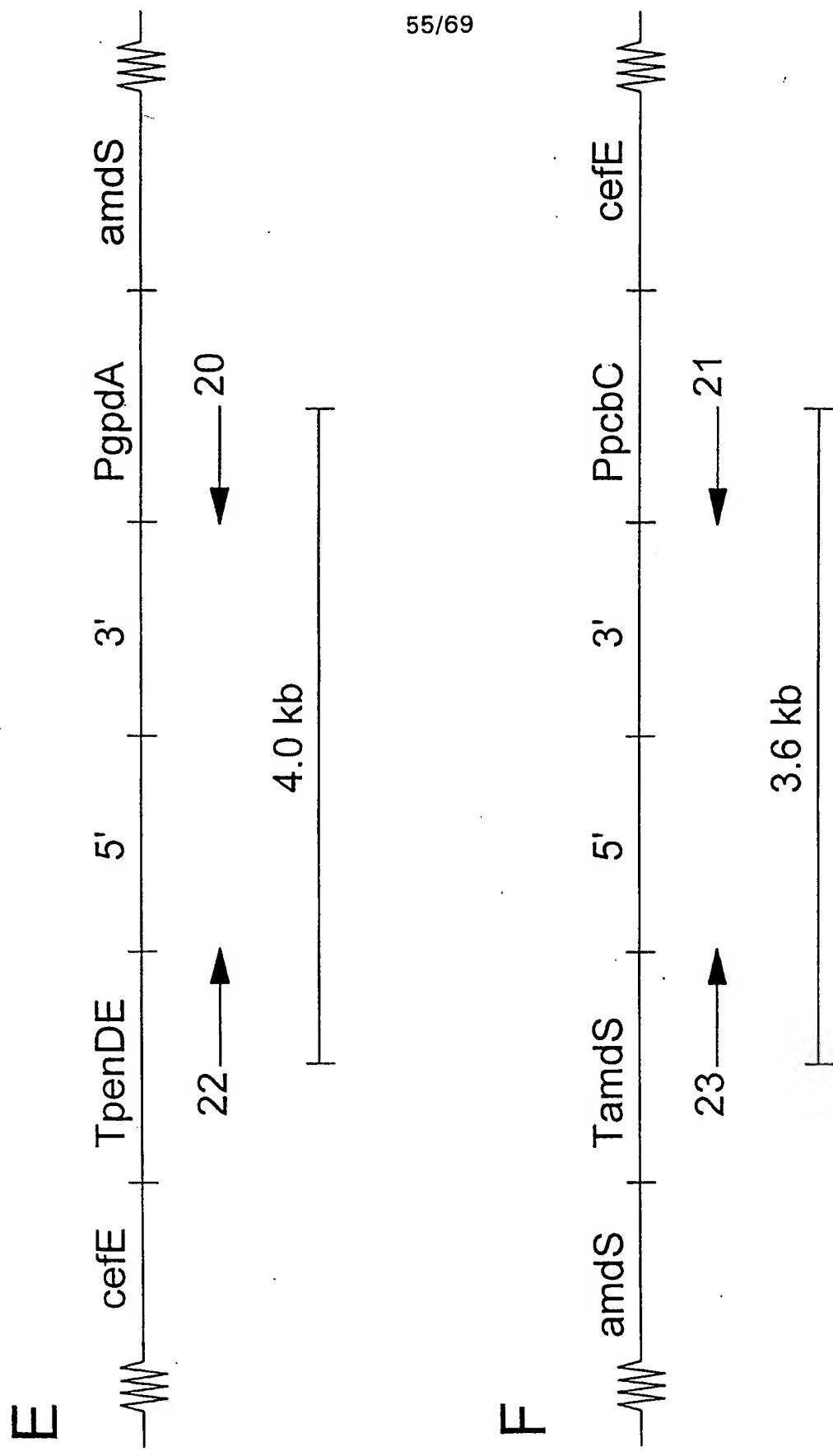


Figures 40A + 40B

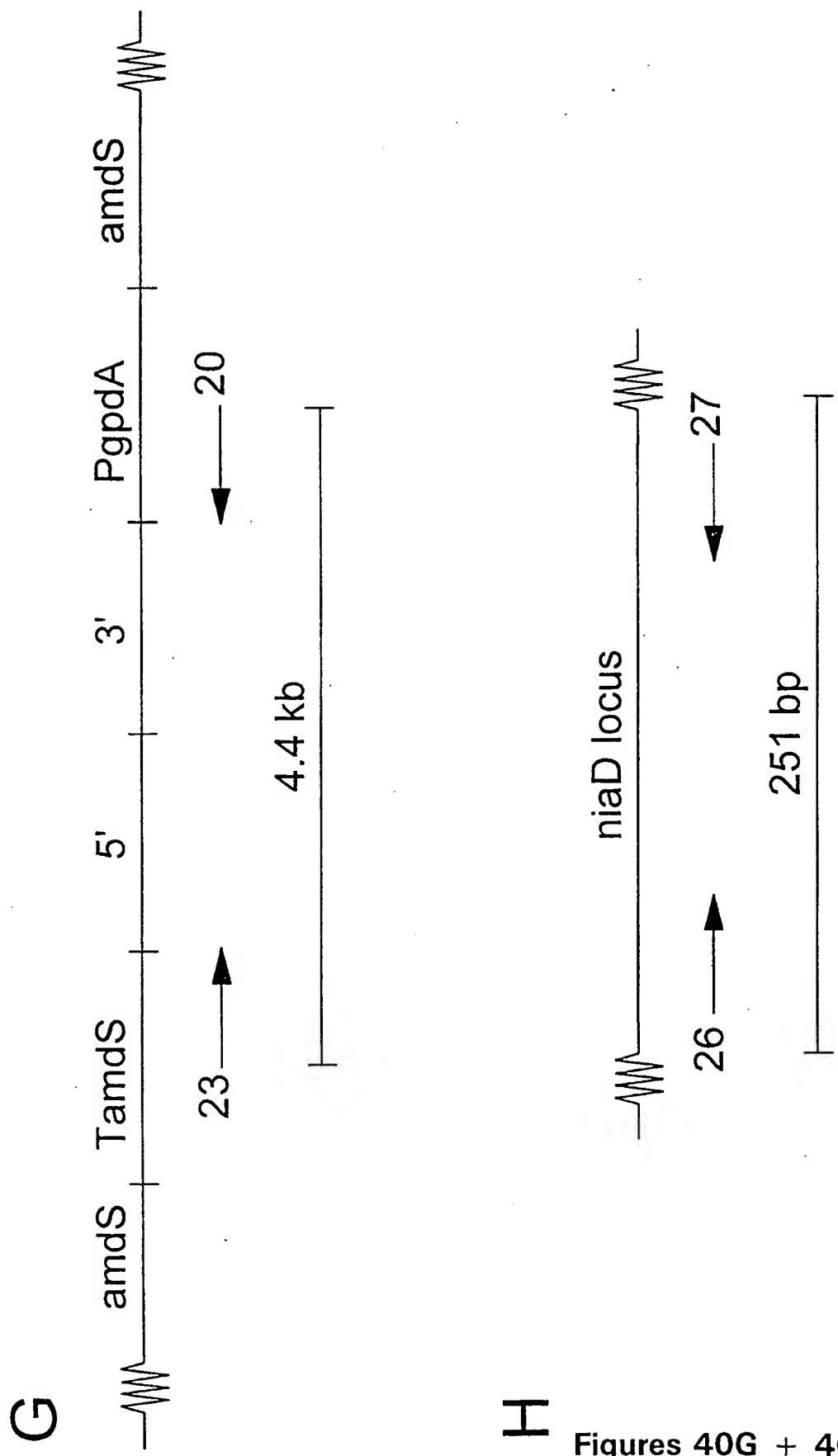


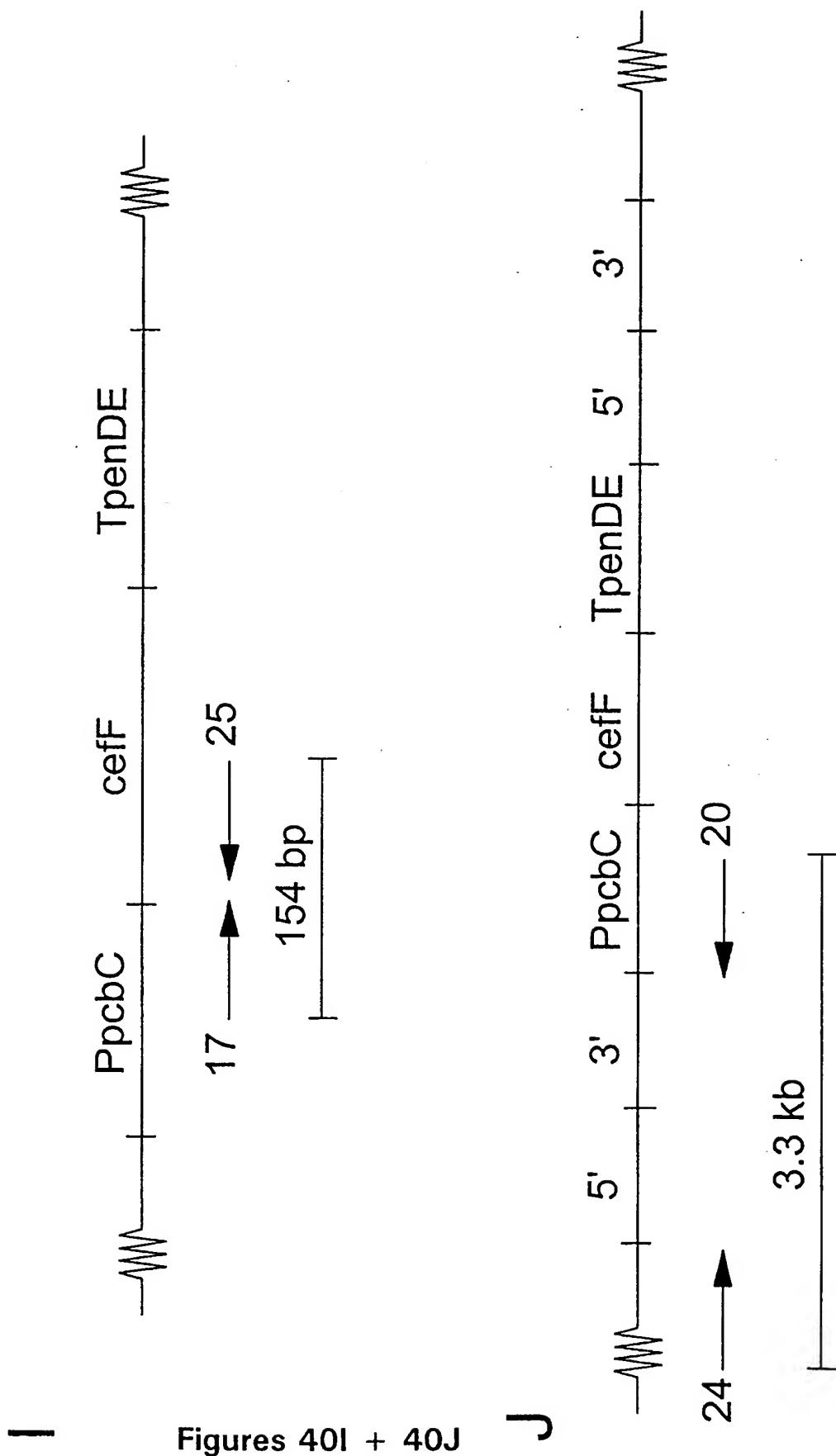
Figures 40C + 40D

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Figures 40E + 40F





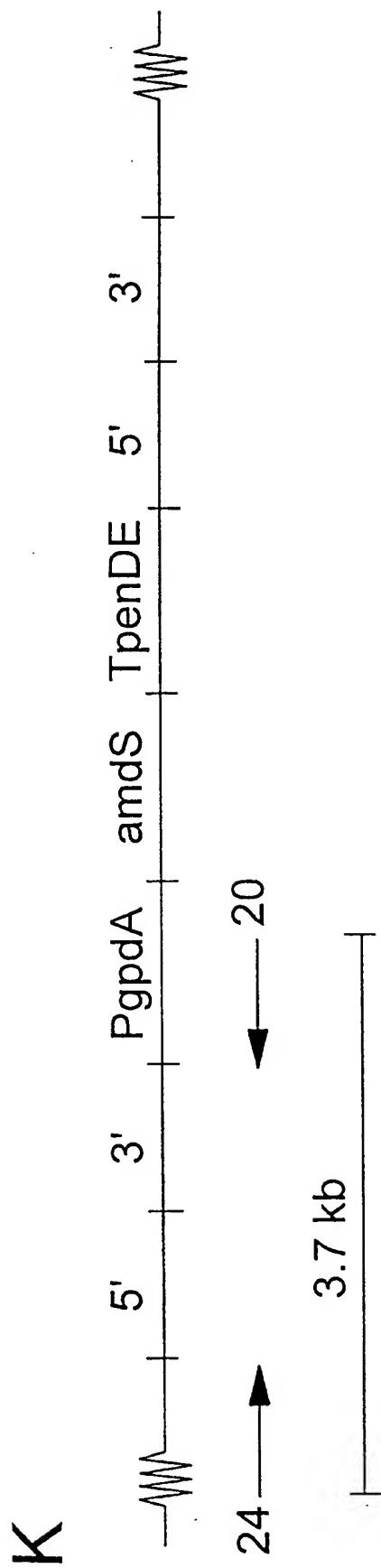


Figure 40K

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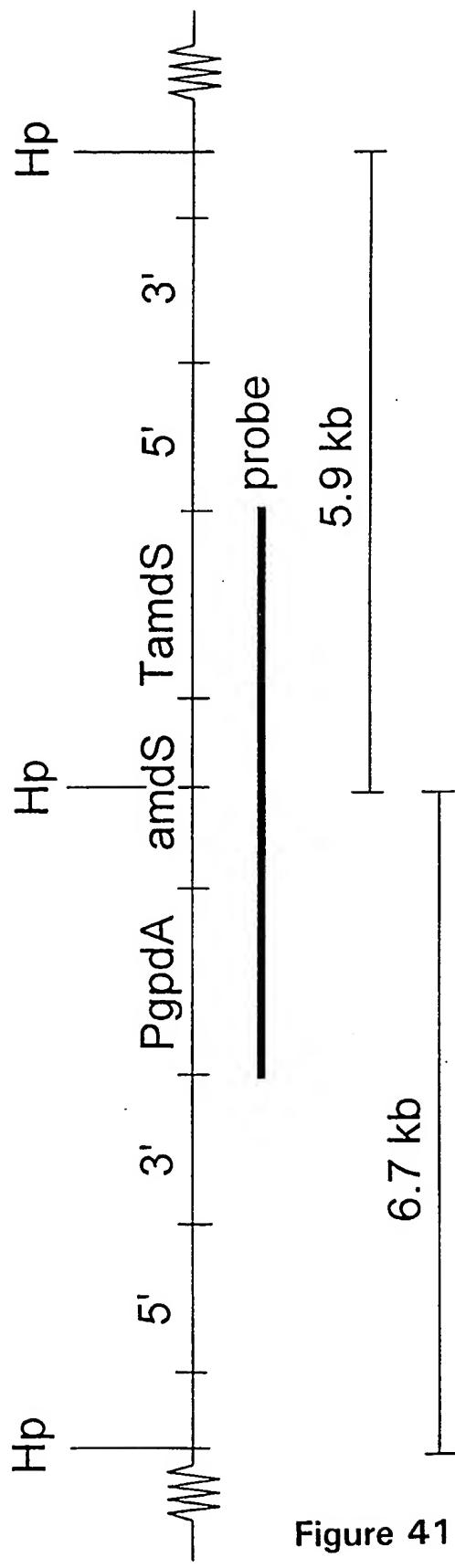
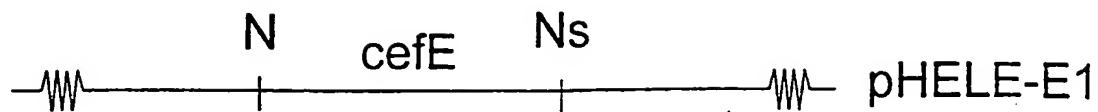


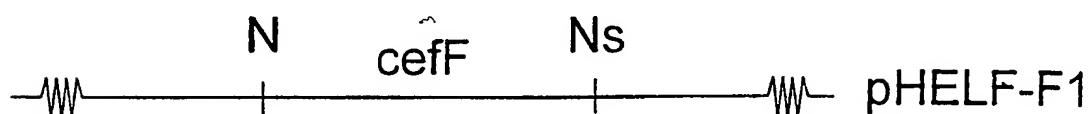
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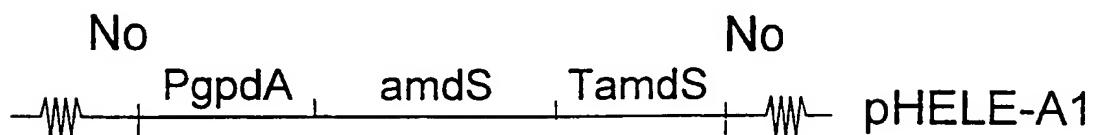
60/69



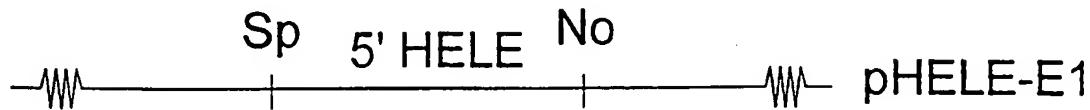
B



C



D



E

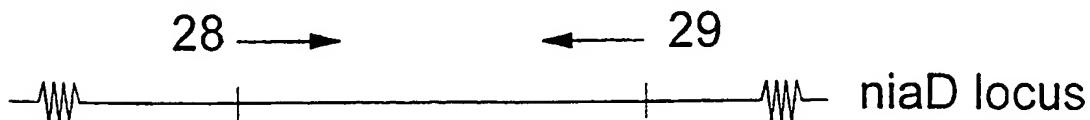


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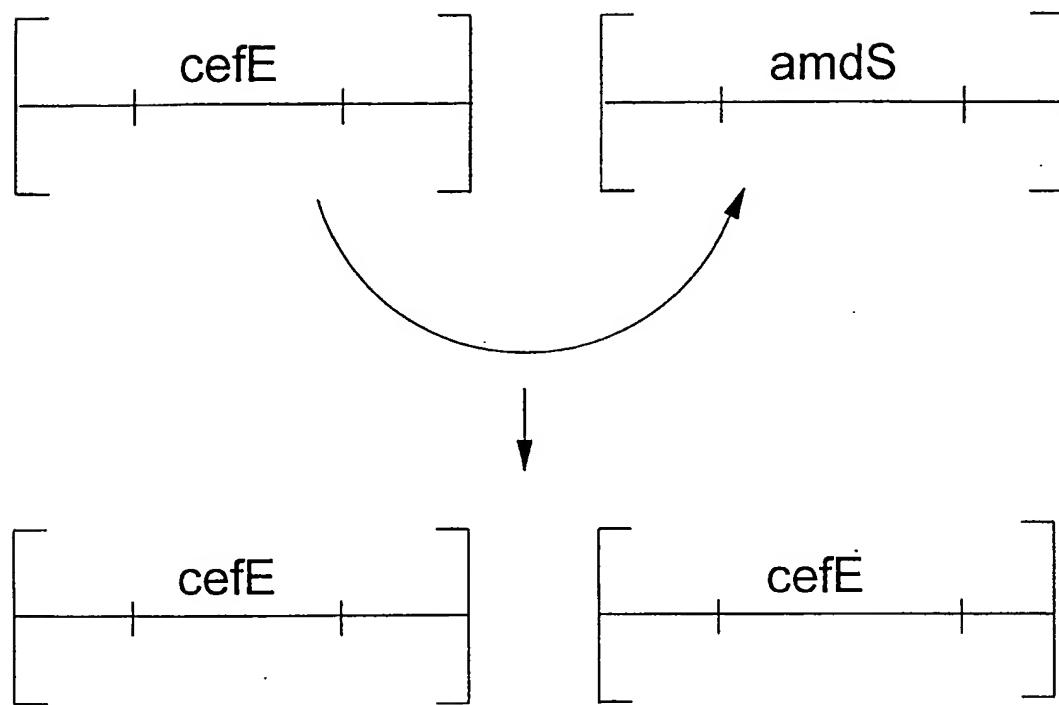


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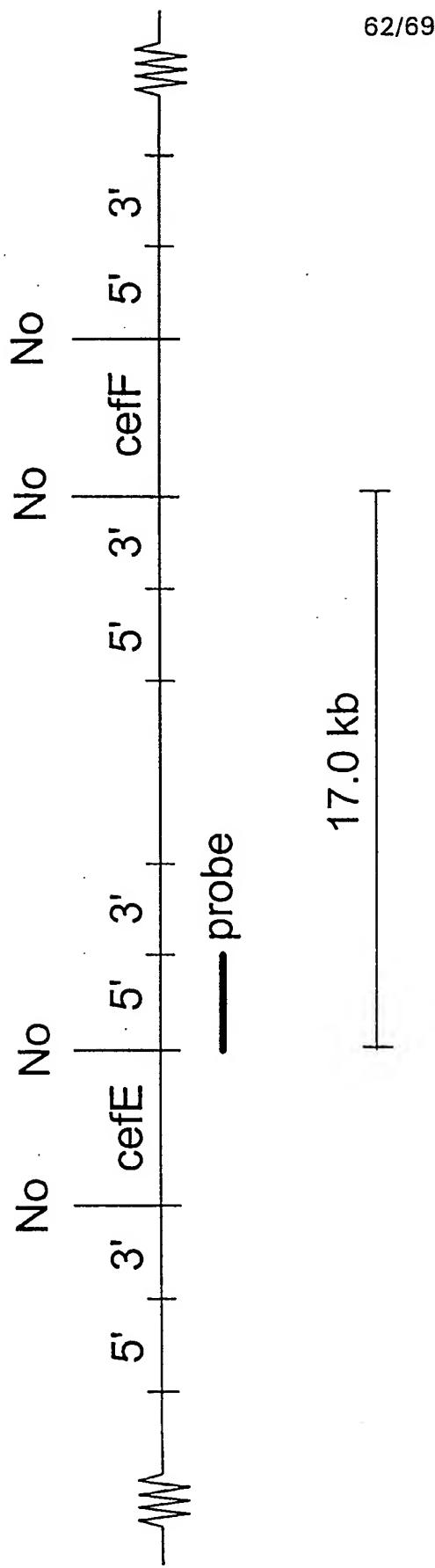
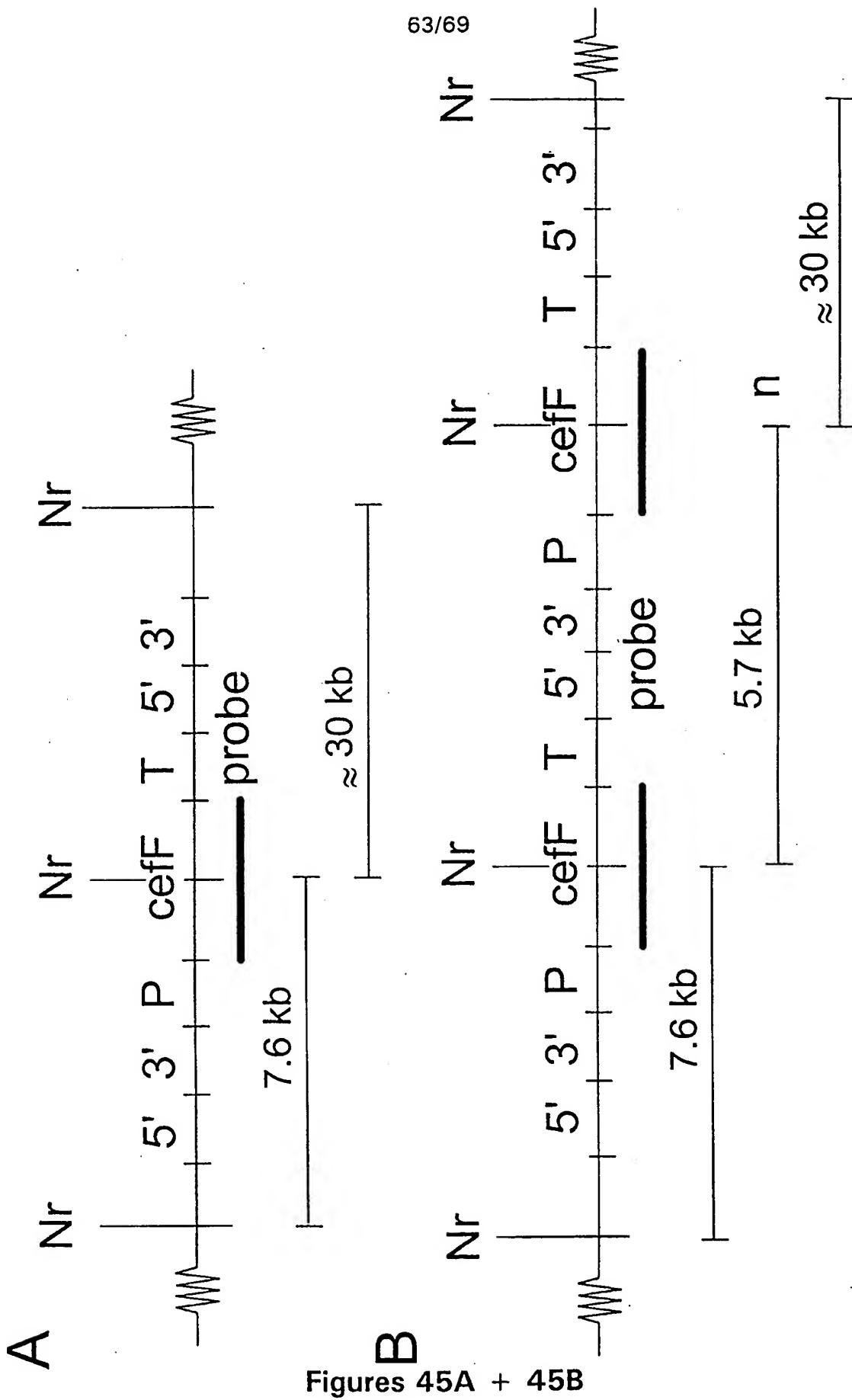
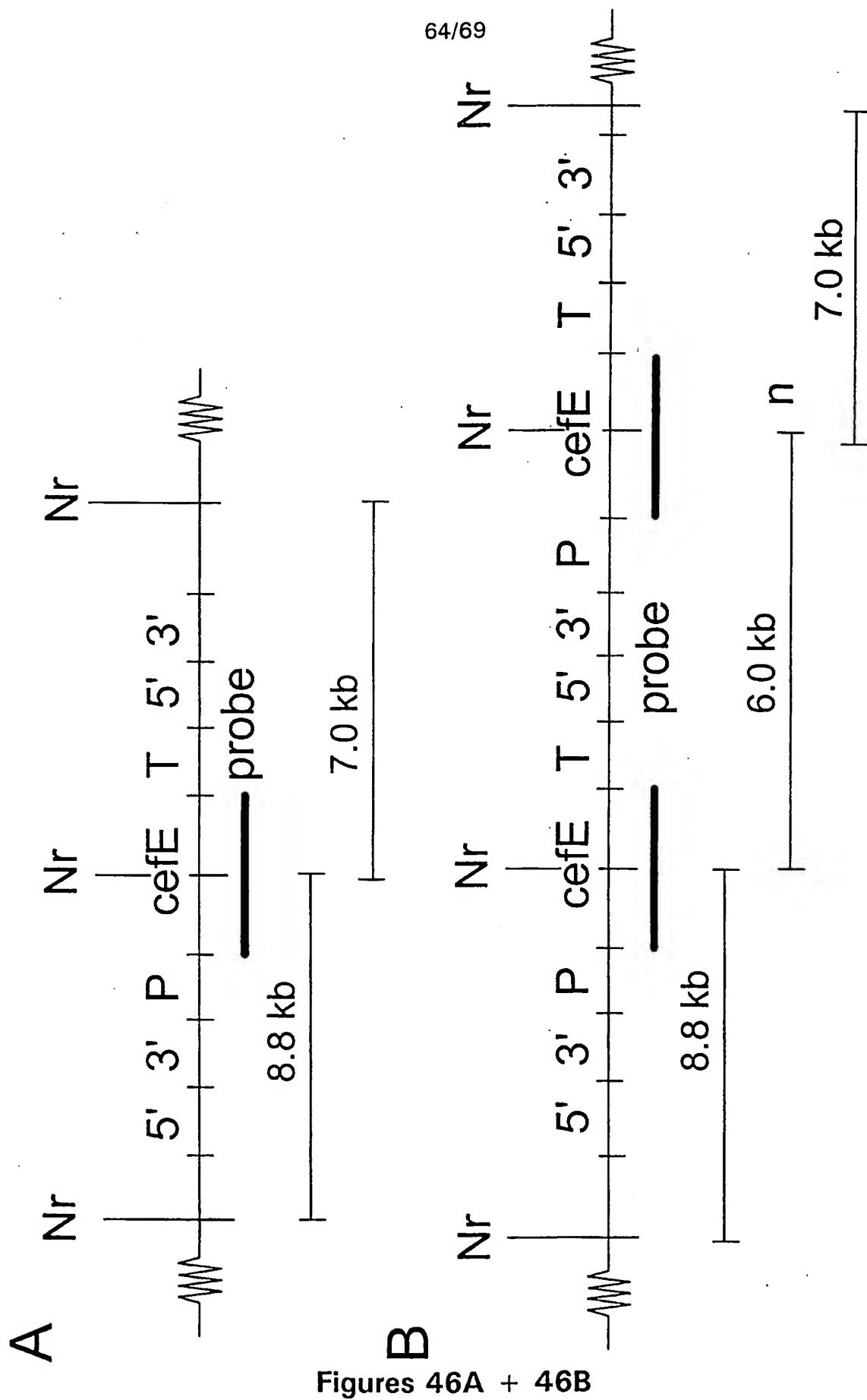
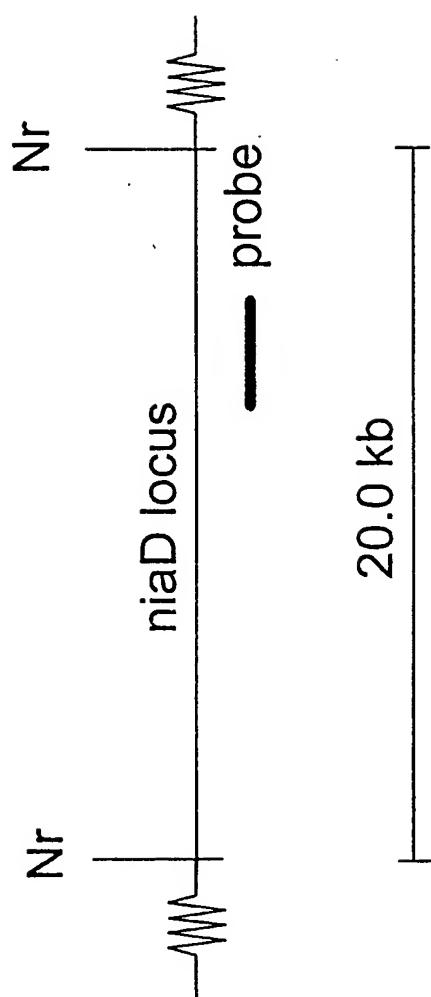


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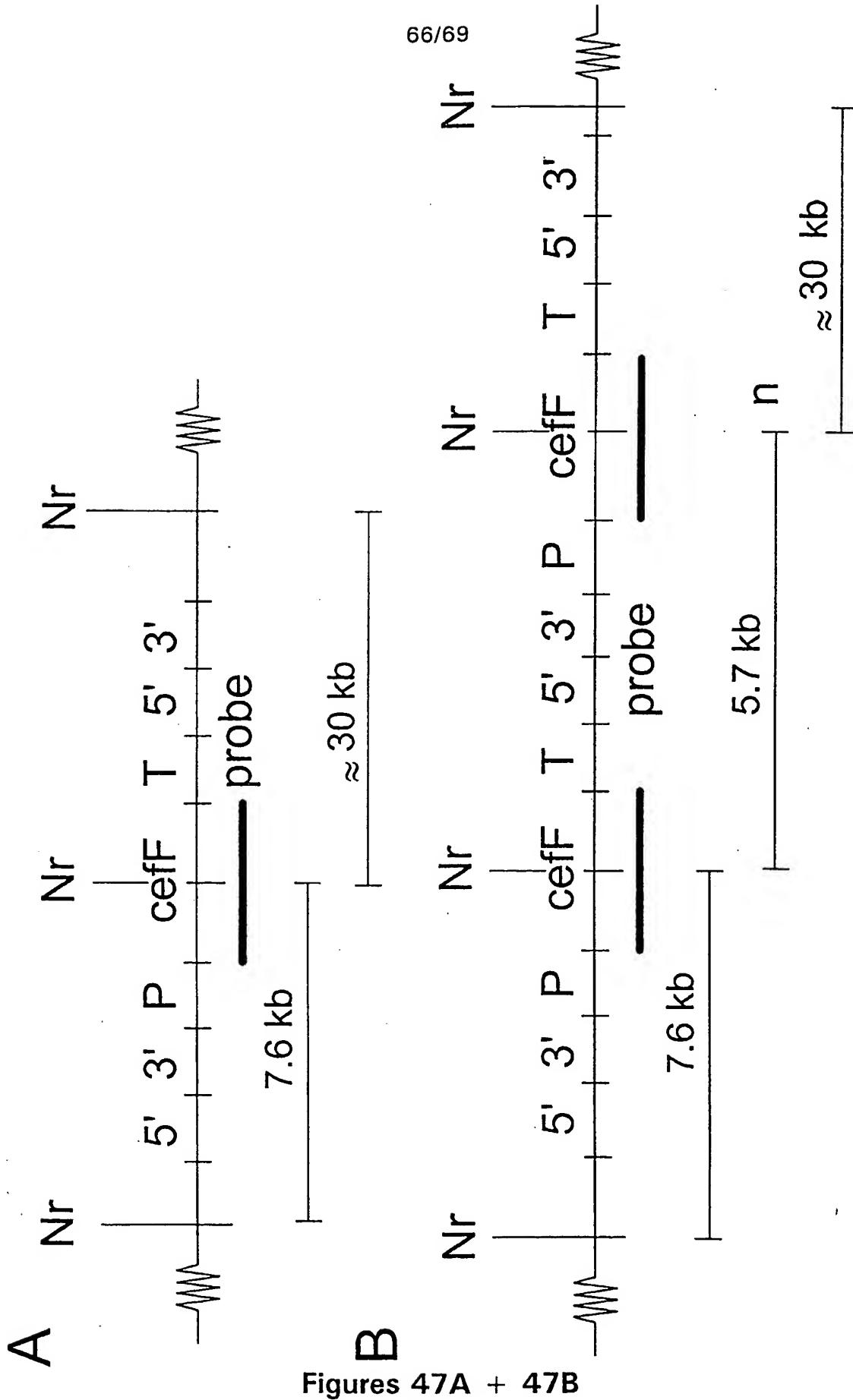


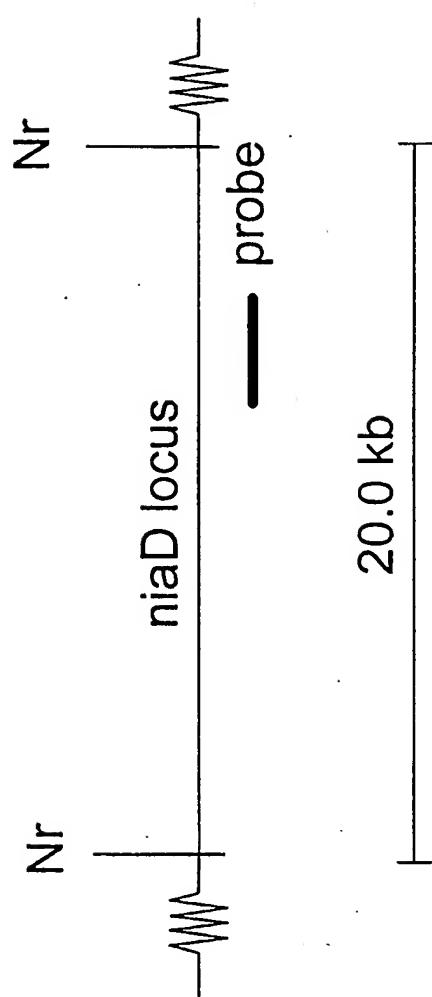




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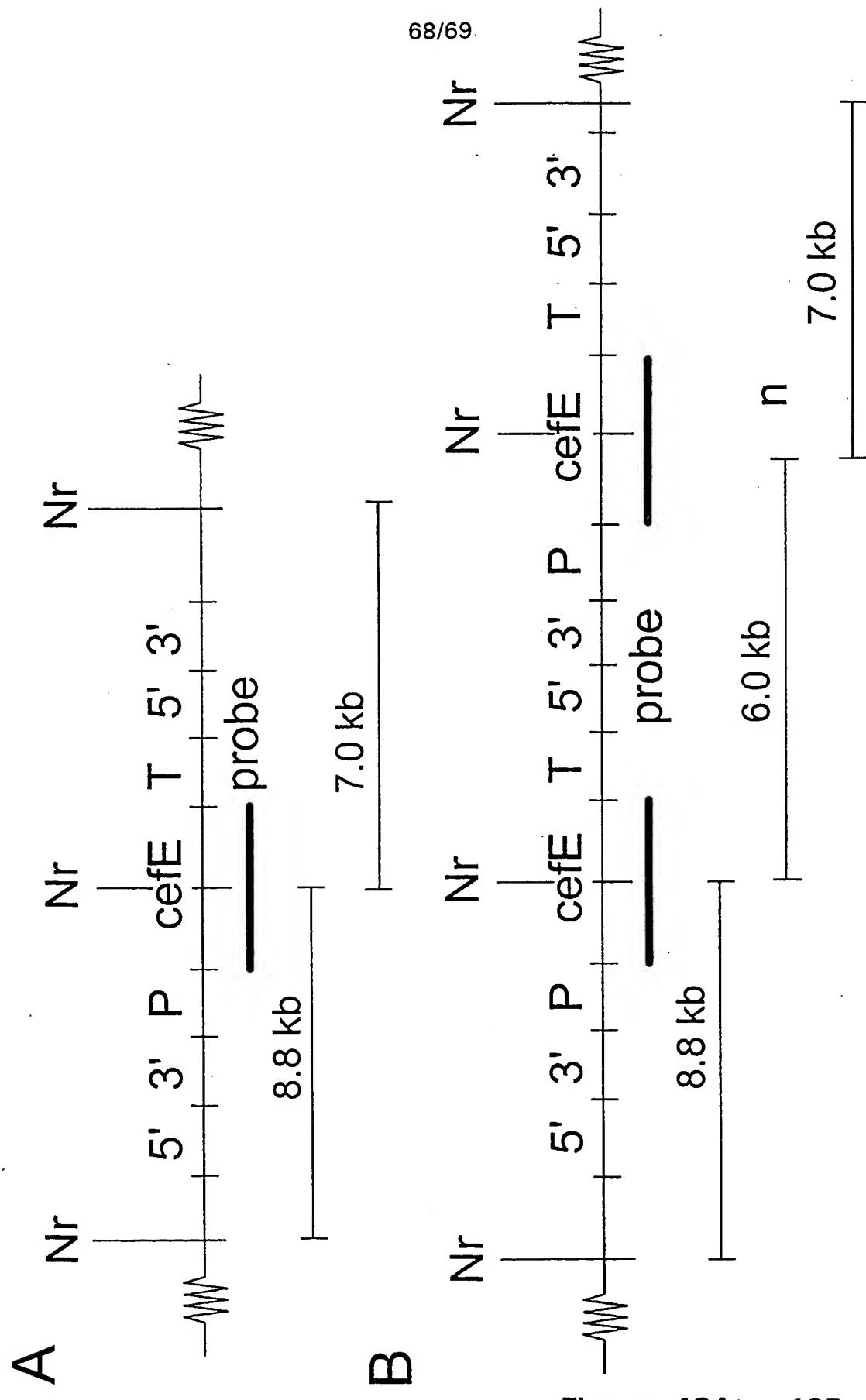
Figure 46C



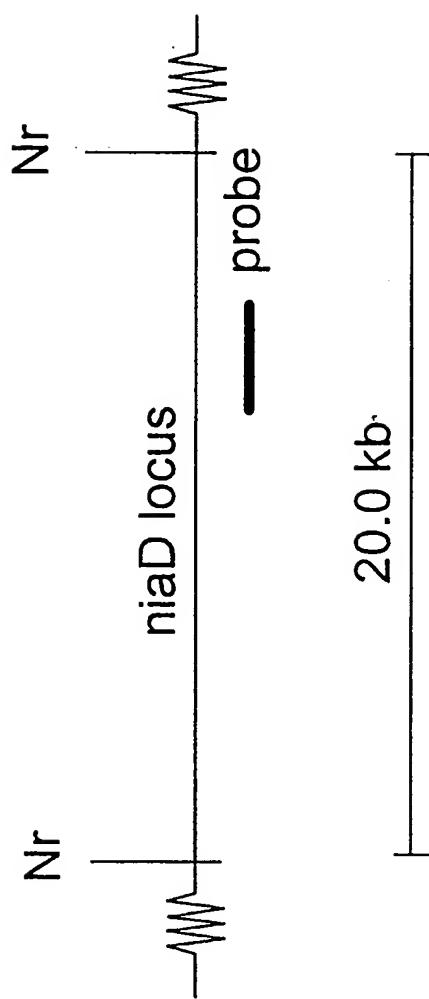


C

Figure 47C



Figures 48A + 48B



C

Figure 48C



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/80, 15/65, 15/52, 1/15		A3	(11) International Publication Number: WO 98/46772 (43) International Publication Date: 22 October 1998 (22.10.98)
<p>(21) International Application Number: PCT/EP98/02070</p> <p>(22) International Filing Date: 9 April 1998 (09.04.98)</p> <p>(30) Priority Data: 97201091.2 11 April 1997 (11.04.97) EP (34) Countries for which the regional or international application was filed: NL et al.</p> <p>(71) Applicant (for all designated States except US): GIST-BROCADES B.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): SELTEN, Gerardus, Cornelis, Maria [NL/NL]; Sterrenweg 81, NL-2651 HZ Berkel En Rodenrijs (NL). SWINKELS, Bart, Willem [NL/NL]; Schutterstraat 5, NL-2611 MX Delft (NL). BOVENBERG, Roelof, Ary, Lans [NL/NL]; 's-Gravenweg 121, NL-3062 ZD Rotterdam (NL).</p> <p>(74) Agent: VOSSIUS & PARTNER; Postfach 86 07 67, D-81634 Munich (DE).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, 'ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 25 February 1999 (25.02.99)</p>	
<p>(54) Title: GENE CONVERSION AS A TOOL FOR THE CONSTRUCTION OF RECOMBINANT INDUSTRIAL FILAMENTOUS FUNGI</p> <p>(57) Abstract</p> <p>The present invention relates to filamentous fungi that comprise in their genomes at least two substantially homologous DNA domains which are suitable for integration of one or more copies of a recombinant DNA molecule and wherein at least two of these DNA domains comprise an integrated copy of a recombinant DNA molecule. The invention also relates to methods for preparing such filamentous fungi and for further multiplying the DNA domains with integrated recombinant DNA molecules through gene conversion or amplification.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02070

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/80 C12N15/65 C12N15/52 C12N1/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VERDOES JC: "Evaluation of molecular and genetic approaches to generate glucoamylase overproducing strains of <i>Aspergillus niger</i> " JOURNAL OF BIOTECHNOLOGY., vol. 36, 1994, pages 165-175, XP002078081 AMSTERDAM NL see the whole document ---	1,3-6, 12,13, 19-21, 23,26,27
Y	FARMAN LM: "Transformation frequencies are enhanced and vector DNA is target during retransformation of <i>Leptosphaeria maculans</i> , a fungal plant pathogen" MOLECULAR AND GENERAL GENETICS, vol. 231, 1992, pages 243-247, XP002078082 BERLIN DE see abstract; figures 1,3 --- -/-	2,7,8, 14,15,22



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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- *P* document published prior to the international filing date but later than the priority date claimed

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

Date of mailing of the international search report

18 September 1998

12.01.1999

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ESPEN, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02070

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 635 574 A (GIST BROCADES NV) 25 January 1995 cited in the application see page 2 - page 3, line 24; claims 20-24; example 1 ---	2,7,8, 14,15,22
Y	WO 95 17513 A (NOVONORDISK AS ;REEH SOLVEJG (DK)) 29 June 1995 see page 6; claims 1-35 see page 1 - page 2 ---	2,7,8, 14,15,22
Y	WO 91 00920 A (UNILEVER PLC ;UNILEVER NV (NL)) 24 January 1991 cited in the application see page 3 - page 8; claims 1-4 ---	2,7,8, 14,15,22
Y	EP 0 758 020 A (GIST BROCADES BV) 12 February 1997 see page 5, line 36 - line 48 ---	2,7,8, 14,15,22
A	EP 0 357 127 A (GIST BROCADES NV) 7 March 1990 ---	
A	VERDOES JC ET AL: "Glucoamylase overexpression in Aspergillus niger: molecular genetic analysis of strains containig mnultiple copies of the glaA gene" TRANSGENIC RESEARCH, vol. 2, 1992, pages 84-92, XP002078083 UK -----	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP 98/02070

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Claims : in part : 1-29

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: in part: 1-29

Aspergillus niger having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

2. Claims: in part: 1-29

Aspergillus oryzae having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

3. Claims: in part: 1-29

Trichoderma (reesei) having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

4. Claims: in part: 1-29

Penicillium (chrysogenum) having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

5. Claims: in part: 1-29

Cephalosporium having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

6. Claims: in part: 1-29

Acremonium having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

7. Claims: in part: 1-29

Fusarium having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

8. Claims: in part: 1-29

Mucor having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

9. Claims: in part: 1-29

Rhizopus having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

10. Claims: in part: 1-29

Phanerochaete having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

11. Claims: in part: 1-29

Neurospora having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

12. Claims: in part: 1-29

Humicola having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

13. Claims: in part: 1-29

Claviceps having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

14. Claims: in part: 1-29

Sordaria having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

15. Claims: in part: 1-29

Ustilago having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

16. Claims: in part: 1-29

Schizophyllum having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

17. Claims: in part: 1-29

Blakeslea having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

18. Claims: in part: 1-29

Mortierella having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

19. Claims: in part: 1-29

Phycomyces having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

20. Claims: in part: 1-29

Polypodium having integrated a recombinant DNA molecule into at least two homologous DNA domains of its chromosome(s), method for preparing this filamentous fungus

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/02070

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